DISSERTATION

REGULATION OF COPPER TRANSPORT INTO AND WITHIN *ARABIDOPSIS THALIANA* CHLOROPLASTS: A FOCUS ON COPPER TRANSPORT PROTEINS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KATHRYN AMY GOGOLIN ENTITLED REGULATION OF COPPER TRANSPORT INTO AND WITHIN *ARABIDOPSIS THALIANA* CHLOROPLASTS: A FOCUS ON COPPER TRANSPORT PROTEINS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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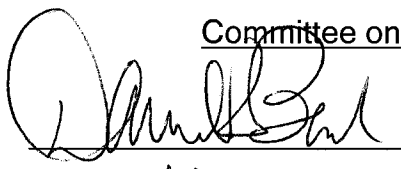

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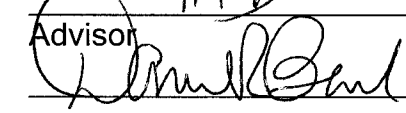

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ABSTRACT OF DISSERTATION

REGULATION OF COPPER TRANSPORT INTO AND WITHIN *ARABIDOPSIS THALIANA* CHLOROPLASTS: A FOCUS ON COPPER TRANSPORT PROTEINS

Copper is an essential micronutrient that is required for the biological processes of photosynthesis and respiration. Nutrients, such as copper, must travel long distances through several organs and across many membranes before they are incorporated into target enzymes. Plastocyanin is a small, copper containing protein that is located within the thylakoid lumen and is vital for photosynthetic activity in higher plants. In addition chloroplasts contain a second target for copper, the superoxide dismutase enzyme CSD2. Although copper is essential it can also be toxic to the cell, therefore there is tight regulation of ion transport. The objective of the research conducted here is to develop a better understanding of copper homeostasis in plant cells. By focusing on the proteins that are involved in the transport of copper new insight can be gained on the delivery pathways of this metal. In this dissertation, I further characterize P-type ATPase of *Arabidopsis* 1 (PAA1) and P-type ATPase of *Arabidopsis* 2 (PAA2). An *Arabidopsis* Copper Chaperone for Cu,Zn Superoxide dimustase (CCS) is identified as a functional homolog of the yeast copper chaperone for Cu,Zn superoxide dimustase (Ccs1/Lys7). I study

the effects of altered CCS expression on copper homeostasis in a plant system and I determine that the Heavy Metal Associated 1 transporter functions to transport a metal other than Cu(I) across the chloroplast envelope which affects photosynthetic activity. Finally, I completed a comprehensive analysis of copper transport protein-protein interactions in *Arabidopsis* studied by the yeast two-hybrid system. With the data gathered here, I propose several new models for copper homeostasis in *Arabidopsis*. I suggest that there is regulation of Fe Superoxide Dismutase (FeSOD), CCS, and CSD2 in the chloroplast which is controlled by metal cofactor availability, specifically copper. By utilizing the yeast two-hybrid technique, I have identified two new possible delivery pathways for copper. I believe that CCS can deliver copper to Heavy Metal Associated 5 to aid in cell detoxification or possible long distance transport of the ion. Additionally, I propose that copper is transported directly from PAA1 to PAA2 in the chloroplast for delivery to plastocyanin.

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Abbreviations used:

ATX1, Antioxidant1; CaMV35S, Cauliflower Mosaic Virus 35S promoter; CCH, Copper Chaperone; CCS, Copper chaperone for Cu/Zn superoxide dismutase; Ctr, Copper transporter; CCP, Copper Chaperone for the Plastid; Col, Columbia-0 Ecotype; COPT1, Copper Transporter 1; CSD1, cytosolic Cu,Zn SOD; CSD2, stromal Cu,Zn SOD; Cu,ZnSOD, Cu,Zn superoxide dismutase; FeSOD, Fe superoxide dismutase; F_v/F_m, the quantum efficiency of open photosystem II centers; GFP, Green Fluorescent Protein; GUS, β-glucoronidase; HMA, Heavy Metal Associated; HSP70, Heat Shock Protein 70; MnSOD, Mn superoxide dismutase; MS, Murashige and Skoog media; MV, methyl viologen; NPQ, non-photochemical quenching; PAA, P-type ATPase of *Arabidopsis*; PC, plastocyanin; PSII, photosystem II; ΦPSII, quantum yield of photosystem II photochemistry; qP, photochemical quenching; RAN1, Responsive-to-antagonist1; SOD, superoxide dismutase; WS, Wassilewskija Ecotype; WT, Wildtype.

CHAPTER 1

Introduction

The goal of the research described in this dissertation was to gain a better understanding about copper transport into and within *Arabidopsis thaliana* chloroplasts by studying the proteins involved in delivery. This introduction chapter discusses currently known copper homeostasis mechanisms that will provide adequate background for the research presented in the following chapters of the dissertation.

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1. Copper in Biology

1.1. Forms and Availability of Copper

Copper is a transitional element, found between nickel and zinc on the periodic table of elements. This metal is atomic number 29 and has an atomic weight of 63.5 [1]. Copper concentrations range from 3-110 ppm in soils and have an average abundance of 55 ppm in the Earth's crust [2,3]. This is quite rare when compared to the elements aluminum (81,300 ppm), iron (50,000 ppm), and manganese (950 ppm). Other elements worth noting due to possible influence on copper transport are: zinc, cobalt, lead, silver, and gold. Zinc abundance is similar to copper with amounts in soils ranging from 16-95 ppm and averages 70 ppm [2,3]. Cobalt and lead are found in similar concentrations, slightly less than copper. Cobalt ranges 2-47 ppm with an average of 25 ppm, whereas lead ranges 7-48 ppm and averages 13 ppm in the Earth's crust. Gold is the least abundant averaging 0.07 ppm [3].

In nature, the common major ores of copper are bornite (Cu_5FeS_4), chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), and malachite ($Cu_2CO_3(OH)_2$). While, cuprite (Cu_2O), covellite (CuS), and native copper (Cu) are found in smaller amounts and are minor ores [4]. Copper has two different oxidation states, Cu^+ (Cu(I)) and Cu^{2+} (Cu(II)) [2]. Copper (I) ions can only be found free in very acidic solutions or complexed with other molecules, whereas free Cu(II) ions are stable in neutral, aqueous solutions that are exposed to the atmosphere [4].

During early geologic time (before 2.5 x 10^9 years ago) both the atmosphere and the oceans were in a reduced state [5]. The dominant species of carbon, nitrogen, and sulfur in this environment would have been CO, CH_4, N_2, NH_3, and H_2S [6]. Metal bioavailability would be limited to Fe, Mn, Zn, Co, Ni, and Mo complexes for integration into molecular cofactors [7]. The first oxygen producers, cyanobacteria, emerged during the Early Archean time period and many scientists believe that these photosynthesizing organisms were the cause of the oxidized atmosphere found during current time [7]. Even though photosynthetic organisms appeared approximately 3.5 x 10^9 years ago, during a transitional period oxygen was only found sporadically in microclimates. It was not until 1.7 x 10^9 years ago that the accumulation of free oxygen resulted in widespread aerobic conditions in the Earth's atmosphere as well as the oceans [5]. Within this newly oxidized environment, iron availability decreased dramatically whereas copper became available as Cu(II) [7]. This change in the availability of copper, is arguable the second most significant event in geologic history after the evolution of oxygen; giving rise to the complex photosynthetic pathway found in modern, higher plant systems.

1.2. Copper and Nutrition

Bioavailability of copper to plants is dependent on the soil type. Copper, especially as Cu(II), has a high affinity to bind to organic matter with an estimated 98% of copper found as a complex in soil solutions [8]. Therefore, organic soils are defined copper deficient if there is less than 20 ppm whereas inorganic soils are deficient if there is less than 4 ppm [2]. Most plants contain

copper concentrations ranging from 5-20 μg/g (ppm) dry weight [8,9]. Symptoms of deficiency start when copper decreases below 5 μg/g dry weight in vegetative tissues, while toxicity levels can be defined as 20 μg/g dry weight or higher in the same tissue [8,9]. The highest quantity of copper can be found in seeds, nuts, and legumes of plants, with some nuts such as cashews and coconuts reaching levels of 30 μg/g or more.

Copper deficient plants can display a wide variety of symptoms depending on the plant species and developmental stage. Symptoms consist of decreased growth rate, distortion or whitening of young leaves, damage to the apical meristem, as well as a decrease in fruit formation [8,10]. Secondary effects of copper deficiency can be a decrease in cell wall formation and lignification in several tissues, including xylem tissue which would result in insufficient water transport [8]. Due to the elevated levels of copper found in reproductive tissue, deficiency has a severe effect on pollen development and viability, fruit and seed production, in addition to embryo development and seed viability. High nitrogen concentrations in soils can induce copper deficiency in plants by decreasing copper mobility and availability [8].

Copper toxicity thresholds vary greatly between species of plants and affect tissues differently depending on metabolic requirements. Excess copper concentrations in the soil tend to decrease root growth before shoot growth due to preferential copper accumulation in that organ [8]. The most common general symptom of toxicity is chlorosis of vegetative tissue. Increased formation of free radicals resulting in oxidative stress can occur when high amounts of Cu, Cd, Pb, or Al are observed at a cellular level [11]. At a

molecular level, photosynthesis is affected by damage to thylakoid membranes resulting in changes to chloroplast ultrastructure, inhibition of electron transport between photosystem II (PSII) and photosystem I (PSI), and impairment of carboxylase and oxygenase activities of RUBISCO [11]. Copper toxicity can also reduce iron up take, even to the point of deficiency, depending on the form of iron available in the soil [8].

1.3. Requirements for Metabolism

The three most abundant trace elements in biochemical systems are iron, zinc, and copper [12]. Since several proteins and enzymes require copper for proper function, copper is essential for survival of most organisms [2]. Copper containing proteins have three major functions: dioxygen transport, catalytic, or copper transport/sequestration [12]. Photosynthetic organisms do not contain copper proteins that function in dioxygen transport. However, one example of such a protein is hemocyanin that can be found in mollusks, arthropods, and annelids. Hemocyanin is an exceptionally large protein (4500 - 9000 kDa) that is oxygenated in the gills of mollusks and transfers oxygen to tissues throughout the organism [12].

Copper proteins that have catalytic functions include oxidation, electron transfer, and superoxide dismutation. Laccase and ascorbate oxidase are both proteins that can oxygenate substrates by transferring four electrons to a dioxygen molecule, resulting in two water molecules [12]. Laccase is a multicopper-containing glycoprotein that has been found in arthropods, fungi, and higher plants. This protein can act as a polyphenol oxidase and has been

most widely implicated in lignin synthesis [13]. In plants, laccases have a predicted N-terminal signal peptide sequence that is for the secretory pathway, which could lead to integration into the cell wall [13]. This is consistent with previous research that has isolated laccase from cell walls.

The biochemical function of ascorbate oxidase is to oxidize alpha ascorbic acid to dehydroascorbic acid [12], however the biological function is not well understood. In plants, ascorbate oxidase is found predominately in the apoplast because dehydroascorbic acid is more readily taken up by the cell than the charged form of the molecule [14]. Ascorbic acid has been highly studied in plants and the major biological function is in oxidative stress defense mechanisms with high levels found in chloroplasts [15]. Even though ascorbate oxidase function is unknown, transcript levels are increased in the light [16]. More recently, it has been suggested that one role could include cell elongation through cell wall loosening mediated by the hormone auxin [17]. Interestingly, plants contain a group of ascorbate oxidases that do not include a copper-binding site. These two groups of oxidases have approximately 25-30% sequence similarity; however it is expected that the proteins lacking copper have a different biological function [18]. Both *Brassica napus* and *Arabidosis* contain ascorbate oxidases lacking copper with functions in *Brassica* involving pollen tube growth [19] and directional root growth in *Arabidopsis* [20].

The most notable electron transfer proteins containing copper are cytochrome *c* oxidase and plastocyanin. Cytochrome *c* oxidase is found in aerobic bacteria and mitochondria of all eukaryotes. This large,

transmembrane protein is the terminal oxidase in the cellular respiration system. The biochemical function of cytochrome *c* oxidase is to use electrons from cytochrome *c* to reduce dioxygen to produce water and at the same time pump protons [21]. The enzyme is located in the mitochondrial inner membrane and contributes to the electrochemical gradient of protons across the membrane. This gradient drives the synthesis of adenosine-5'-triphosphate (ATP), which is the primary energy source for living organisms. Most cytochrome *c* oxidases have three core subunits that are highly conserved across organisms. There are two copper binding sites (Cu_A and Cu_B) with a total of three copper atoms [22]. The large C-terminal domain of subunit II in the intermembrane space contains a Cu_A-center that functions as an electron conductor. While subunit I is the site of oxygen reduction, containing two atoms of copper at the Cu_B site and two heme groups [22].

Plastocyanin is a small, blue, copper-containing protein found in photosynthetic organisms. Defined as a blue protein because of the form of copper bound (Cu II) and the characteristics of the binding site which consists of two histidines, one cysteine, and one methionine [12]. Plastocyanin contains one copper ion, is approximately 10 kDa, and is located inside the lumen of plant chloroplasts as well as some cyanobacteria and green algae. The biochemical function is to transfer electrons between cytochrome b_6f complex and PSI in the light-mediated reaction of photosynthesis [11]. The structure of plastocyanin is an eight strand, antiparallel β-barrel [23] and the copper binding site is highly conserved across higher plant systems as well as some algae at HIS^{42}, CIS^{92}, HIS^{95}, and MET^{100} [11]. The photosynthetic machinery of

photosystem II and photosystem I are located within the membrane of the thylakoid. Similar to cellular respiration in mitochondria, this machinery drives an electrochemical gradient that produces ATP as well as the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). The products from this light-dependent pathway are then used to convert carbon dioxide to sugar, for the plant to use as food [11]. This carbon fixation, via the Calvin cycle, occurs in the stroma of the chloroplast.

The third type of catalytic copper protein is the superoxide dismutase enzyme which requires a metal cofactor for proper function. There are three different kinds of superoxide dismutases, manganese, iron, and copper, zinc [2]. This enzyme is responsible for reducing oxidative stress caused by superoxides (O_2^-) and highly reactive hydroxyl radicals (OH) within the cell by mediating the following reaction:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide (H_2O_2) is then converted to H_2O via catalases and peroxidases [24,25]. Manganese and iron superoxide dismutases (SOD) are structurally similar with the metal ion not having a structural role. Copper, zinc SOD has two equal subunits with each subunit containing both one copper and one zinc ion [12]. The presence of types of SOD varies between species, but many plants have a mitochondrial MnSOD, a cytosolic CuZnSOD, and a chloroplastic FeSOD and/or CuZnSOD [24]. In plants, all three kinds of SOD are nuclear encoded with targeting sequences that directed them to their subcellular locations.

The last major group of copper containing proteins, and the focus of this dissertation, functions in copper transport and sequestration. This group of proteins will be described in detail in section 2.1. Types of Copper Transport Proteins.

2. The Biochemistry of Copper Transport

2.1. Types of Copper Transport Proteins

Copper uptake and distribution is tightly regulated because free copper ions are very toxic, even at low levels. The presence of free copper ions (Cu(I) or Cu(II)) in the cell can cause autooxidation of proteins, lipids, and nucleic acids [26]. It is speculated that intracellular free copper concentrations are very low because of the binding capacity of proteins that can sequester and traffic these metal ions [27]. The three major categories of copper homeostasis proteins are membrane transporters, copper chaperones, and metallothioneins.

Currently, it is unknown which form of copper is generally taken up by different organisms however experimental data in yeast suggests copper is imported as Cu(I) [28-30]. Some organisms such as yeast contain metalloreductases on the plasma membrane to reduce Cu(II) to Cu(I) extracellularly before transport [31,32]. Once copper has been reduced, there are several different types of transporters that can transport Cu(I) across cell membranes. One common type of transporters is the copper transporter family (Ctr) first discovered in plants but most studied in yeast. Members of this family

are predicted to have three transmembrane domains and are rich in Met and Cys/His motifs (Figure 1A) [33-35]. Depending on the organism, the Met motifs can vary between MxM, MxxM, and MxMxM, but are generally found as repeat motifs at the amino terminus. Sequences that are rich in cysteine and histidine can be found at the carboxy terminus which is thought to be in the cytosol [34,35]. In addition to yeast, copper transporters (Ctr) are found in a wide variety of eukaryotic organisms ranging from plants to mammals, including *Arabidopsis* (COPT family) [36].

A second group of membrane transporters is a diverse superfamily of P-type ATPases found in virtually every kind of organism. The membrane transporters that comprise this large group are categorized by their ion specificity [37,38]. The Heavy Metal Associated (HMA) Family of ion transporters is classified in the P_{1B}-type ATPase sub-family. This group is predicted to have eight transmembrane domains with a large cytosolic loop between the sixth and seventh transmembrane domain (Figure 1B) [39,40]. There is a heavy metal binding domain at the amino and/or carboxy termini as well as a CPx motif in the sixth transmembrane domain that is thought to function in ion transduction [41,42]. Another conserved sequence is DKTGT, found in the large cytosolic loop, which is the site of phosphorylation. Once a metal binds to the transporter, ATP is utilized for phosphorylation that changes the conformation of the protein resulting in the translocation of an ion across the membrane [42]. The P-type ATPase superfamily transporters are not only found cell membranes but also within the cell in organelle membranes, specifically the Golgi complex and the chloroplast.

Lastly in gram negative bacteria, there is an efflux pump used to transport copper out of cells. The four-component pump found in *E. coli* that spans the inner membrane, periplasm, and outer membrane. Since *E. coli* is thought to diffuse Cu(I) through it's cell membrane, this bacteria has most likely evolved this periplasmic export system as a defense to copper toxicity [43].

In addition to transporters, organisms also have proteins that shuttle Cu(I) from one place to another within the cell, called metallochaperones. Unlike other chaperones, these proteins do not aid in protein folding [44]. Instead, metallochaperones for copper (referred to as copper chaperones from this point forward) are small, low molecular weight, intracellular proteins that carry Cu(I) (or other metal ions) from one target to another [45]. It is believed that copper chaperones are highly target-specific, always trafficking Cu(I) to and from particular proteins [26]. The crystal structure of the yeast ATX1 (Antioxidant1) chaperone shows a βαββαβ (ferredoxin-like) fold that is a conserved pattern among ATX1 homologs as well as some other copper homestasis proteins including P_{1B}-type ATPase transporters (Figure 2) [46,47]. Most ATX-like copper chaperones contain a putative metal binding motif, CxxC, near or in the first alpha helix [44,48]. Another type of copper chaperone is the Copper Chaperone for Cu,Zn Superoxide dimutase (CCS) protein that has been found in yeast, insects, plants, and humans that functions to transport copper to cellular superoxide dismutase enzymes [49,50]. Crystallographic structure analyses of CCS proteins indicate that it consists of three domains: an ATX-like domain (domain I), a SOD-like domain (domain II),

and a unique domain (domain III) [51-53]. A CxxC metal binding sequence is present in the amino terminal, ATX-like domain of this copper trafficking protein [52].

The third major type of copper homeostasis proteins are metallothioneins which are small, cysteine rich proteins that have been found in a variety of different organisms. Even though these proteins are relatively small their sequence can contain up to 30% cysteine residues [54]. Some metallothioneins are able to bind approximately 12 copper ions, six in the β domain and 6 in the α domain [55]. With this high binding capacity for copper, metallothioneins have the potential to sequester a large amount of excess metal in the cell. *Arabidopsis* contains four genes encoding metallothioneins that comprise two groups classified based on their structure, MT1 and MT2 [56]. There is some evidence that increased mRNA levels of MT2 are correlated with copper tolerance in some ecotypes of *Arabidopsis* [57]. More recent experimental data suggests that metallothioneins in *Arabidopsis* have specific function. GUS fusion analysis has shown that *MT1a* and *MT2b* are involved in copper transport in the phloem, while *MT2a* and *MT3* sequester excess metal ions in mesophyll cell and root tips [58].

2.2. Metal Related Interactions and Specificity

Metal binding sequences generally include cyteine and/or histidine residues in the following motifs: CxxC, CCxSE, His-rich (including Hx and H repeats), Cys-rich, and Cys/His-rich [42,59]. This is especially true for P_{1B}-type ATPase transporters, but these motifs can also be found in copper chaperones

and metallothioneins. Several protein-protein interactions between these transporters and copper chaperones have been observed using yeast 2-hybrid assays as well as biochemical metal transfer assays [60-63]. The P_{1B}-type ATPase subgroup of transporters is further divided into two different groups based on the putative heavy metals they are hypothesized to transport. The first group is the $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ ion transporters and consists of HMA1, HMA2, HMA3, and HMA4 in *Arabidopsis*; while the second group includes HMA5, PAA1 (HMA6), RAN1 (HMA7), and PAA2 (HMA8) and is thought to transport Cu^{2+}/Ag^{2+} ions [38,64]. These two groups were originally developed based on sequence and phylogenetic analysis with the transporters in the Zn group containing histidine rich regions in the amino and/or carboxy termini areas of the proteins, whereas the transporters in the Cu group have one or two MxCxxC heavy metal binding domains in their amino terminus region. There has been a significant amount of experimental data that supports the classification of these two groups, however more recently there has been some implication that HMA1 could be a copper transporter rather than a Zn transporter as hypothesized [65]. The protein sequence of HMA1 is slightly different from HMA2, 3, and 4 in that the hisitidine rich region is in the amino terminus and it has a rather short carboxy terminus. These differences between the HMA1 transporter and others in the Zn or Cu groups have created much dispute over the function of this protein.

Metal specificity seems to rely on both positions of conserved amino acid residues as well as the conformational structure of each protein. Recently, it has been suggested that sequences within the sixth, seventh, and eighth

transmembrane domains in the P_{1B}-type ATPase transporters contribute to metal specificity [42]. Copper ions have an affinity to bind to sulfur or nitrogen ligands in histidine, methionine, and/or cysteine amino acids. This is true in plastocyanin where copper is bound to two nitrogen ligands in two histidine residues and two sulfur ligands, one in each cysteine and methionine (Figure 3A) [12]. In copper trafficking proteins that have a conserved MxCxxC heavy metal binding domain, copper coordination is theorized to be isolated to two sulfur ligands in the two cysteine residues (Figure 3B) [66]. Structure analysis of the yeast ATX1 bound with Hg(II) and a domain of the Menkes disease protein (Mnk4) bound with Ag(I) indicate that the conserved methionine residue is not in close enough proximity to interact with the metal ion [47,67]. In yeast 2-hybrid experiments it has been observed that when several conserved lysine residues located near the MxCxxC domain are mutated in the yeast ATX1 copper chaperone it losses interaction with the Ccc2 transporter [68]. This implies that these lysines are necessary for the two proteins to interact with each other or alters the conformation of the metal binding pocket.

3. Copper Transport Proteins across Heterotrophic Models

Since many aspects of copper homeostasis are conserved across organisms, the following sections are a review of what is known about copper trafficking across selected model systems: bacteria, yeast, cyanobacteria, and plants, the focus of this dissertation. For a complete list of copper trafficking

proteins and targets in these various organisms, please refer to Tables 1-4 at the end of the chapter.

3.1. Bacterial Copper Transport Mechanisms

Many transporters, copper chaperones, and target enzymes for copper present in *E. coli* can be found in other prokaryotes, as well as more complex eukaryotic systems. *Enterococcus hirae* is one model organism for researching copper transport in a prokaryotic system; as a result it is vastly studied and well understood [69-71]. This Gram-positive bacteria contains two P-type ATPase transporters, located in the plasma membrane, that function in copper transport (Figure 4A) [72,73]. The expression of both CopA and CopB transporters are inducible under high extracellular copper concentrations, however CopB mutants show significantly increased cellular copper levels [73]. This suggests that CopA is an ion importer, while CopB functions as an efflux mechanism. Intracellular levels of copper are regulated by a *cop* operon that consists of four genes: *copA, copB, copY, copZ*. The genes *copA* and *copB* encode the two copper transporters, while *copY* acts as a repressor and *copZ* functions as an activator [74]. Although currently unknown, it is thought that *Enterococcus hirae* contains an extracellular reductase to convert Cu(II) to Cu(I) for cellular uptake of copper. Once copper enters the cell through the CopA transporter, a copper chaperone called CopZ shuttles Cu(I) to CopY, a transcriptional repressor [74,75]. The current model for copper regulation can be summarized as follows: when zinc is bound to CopY it represses transcription of the *cop* operon, however if CopZ delivers copper to CopY, the

repressor disassociates from the promoter allowing transcription to occur [71,76]. Structural analysis of the CopZ chaperone, via NMR spectroscopy, shows that in addition to the typical metal binding domain (MxCxxC) this protein exhibits a βαββαβ ATX-like folding pattern [77].

A second prokaryotic model for studying copper transport is the bacteria *Escherichia coli*. While copper homeostasis is not well known in this Gram-negative bacteria, targets for copper include a Cu,Zn SOD in the periplasm [78,79] as well as cytochrome bo_3 that is classified as a heme-copper oxidase (Figure 4B) [80]. Although there is the requirement of copper for proper function of these enzymes, most of the components that make up the known copper trafficking system in *E. coli* function in detoxification. Copper (II) ions seem to pass through the outer membrane via porins and are then reduced to Cu(I) by the copper reductase NDH-2 [81,82]. Copper, as Cu(I), can then perhaps diffuse through the cytoplasmic (inner) membrane [83]. The P-type ATPase transporter, CopA, is located in the inner membrane and functions as an efflux transporter, detoxifying the cytoplasm from copper ions [84]. Also located in the periplasm is a multi-copper oxidase, CueO, which has similarly been demonstrated to aid in copper detoxification [85]. The expression of both the CopA and CueO proteins are regulated by a cytoplasmic copper sensing protein called CueR [86,87]. Another efflux mechanism for copper is a four-component pump, CusCFBA, which spans the inner membrane, periplasm, and outer membrane [88,89]. Expression of this pump, in part, is regulated by a two-component signal transduction system (*cusRS*), that involves a membrane bound histidine kinase (CusS) and a cytoplasmic reponse regulator

(CusR) [88]. The CusF constituent of the efflux pump is a copper chaperone found in the periplasm that can traffic Cu(I) to the CusCBA channel for removal through the outer membrane [89].

3.2. A Eukaryotic Model for Copper Regulation: *Sacchaomyces cerevisae*

With the presence of internal organelles, yeast and plants have a similar copper regulatory system which includes several direct homologs. Yeast has a copper reductase protein complex, Fre1/Fre2, that is located extracellularly to reduce Cu(II) to Cu(I) for uptake (Figure 5) [31,32]. A total of three Ctr (Copper transporter) proteins have been discovered in yeast. Two of these transporters, Ctr1 and Ctr3, are located in the plasma membrane and data suggests that they are high affinity Cu(I) importers [29,30]. The *CTR1, CTR3*, and *FRE1* genes are under the regulation of the copper-sensing MAC1 transcriptional factor [31,90-92]. Copper deprivation induces a signal transduction system in which MAC1 binds to the promoter elements of the copper reductase and copper transporter genes starting transcription [91].

The cellular localization of the Ctr2 transporter has been debated in the past; however recent evidence indicates that it is incorporated into the vacuolar membrane where it releases copper back into the cytosol when concentrations are critically low [93-95]. In addition to the Ctr family of transporters *S. cerevisae* has a P-type ATPase transporter, Ccc2, located in the Golgi [96] and is thought to translocate copper into the endomembrane system. The ATX1 (Antioxidant1) protein has been identified as the cytosolic copper chaperone for the Ccc2 transporter [97,98]. Iron metabolism has been

linked to copper homeostasis in yeast through a copper requiring oxidase, Fet3, needed for ferrous iron uptake that is the target for copper in the endomembrane system [30,99-101].

Copper transport to the mitochondrion for integration into cytochrome *c* oxidase (Cco) in yeast has been highly studied, although it is not yet fully understood. Two putative chaperones, Cox17 and Cox 19, have been implicated in cytosolic transport because of dual localization to the cytoplasm and intermitochondrial membrane space [102,103]. However, due to biochemical and mutant analysis both proteins have been eliminated as the chaperones for mitochondrial shuttling [104]. As a result, it is currently unknown which protein is responsible for copper delivery to the mitochondrion. Once copper is in the intermitochondrial membrane space several proteins are required to work in concert to incorporate the ions into Cco. The existing model is, Cox 17 donates copper to Sco1 and Cox11 [105], which then results in the integration of copper into the Cu_A site in Cox2 subunit and Cu_B site in the Cox1 subunit of cytochrome oxidase, respectively [106,107].

Lastly, the CCS copper chaperone is the protein for ion shuttle to a copper-requiring SOD (SOD1) [108,109]. In addition to the cytosol, a small fraction of both SOD1 and CCS can be found in the intermitochondrial space even though there is no presequence for targeting to the mitochondria [110]. Yeast also contain two known metellothionein-like proteins, Cup1 and Crs5, which can sequester excess copper thereby decreasing cell toxicity [111,112]. Whereas the sequence homology between the Crs5 peptide and mammalian metallothioneins is high, there is little homology between the Crs5 and Cup1

proteins [112]. A transcriptional regulator, Ace1, has been identified that can activate transcription of Cup1 and Crs5 metallothioneins [112,113], as well as the SOD1 enzyme [114] in the presence of copper.

4. Copper Transport Proteins across Autotrophic Models

4.1. Copper Transport in Photosynthetic Cyanobacteria and Algae

The photosynthetic machinery and process in cyanobacteria is similar to plants, especially under sufficient copper conditions. Therefore, studying the mechanisms and regulation of copper within cyanobacteria can help to understand chloroplastic homeostasis in higher plants. Cyanobacteria contain internal thylakoid membranes, which are the site of electron transport. There are two major targets for copper within cyanobacteria, plastocyanin (PC) and cytochrome oxidase (CO), which are both found in the thylakoid (Figure 6) [115,116]. In an environment deprived of copper, some cyanobacteria can use the iron containing cytochrome c_6 protein as a terminal electron acceptor in photosynthesis instead of the copper-requiring protein plastocyanin [117]. Copper is transported through the cell membrane and the thylakoid membrane by two P-type ATPases called CtaA and PacS, respectively [118,119]. A copper chaperone similar to the yeast ATX protein has been identified in the model organism *Synechocystis* PCC 6803 that interacts in bacterial 2-hybrid experiments with both of the P-type ATPase transporters [63]. This suggests that once copper enters the cell through CtaA it is shuttled to PacS via ATX1 in the cytoplasm, for plastocyanin or cytochrome oxidase targets.

Chlamydomonas is a highly studied unicellular, flagellate, green alga. Cells of Chlamydomonas contain one large chloroplast that has similar photosynthetic machinery as higher plants. There are three target enzymes that require copper for proper function in Chlamydomonas, cytochrome oxidase, plastocyanin, and multicopper oxidases involved in iron acquisition [120]. It has been proposed that copper reductases work in concert with a copper transporter to import the metal into cells. A COPT1 transporter protein has been identified in Chlamydomonas which is similar to the *Arabidopsis* COPT1 [121]. In addition, this organism contains three different HMA transporters (HMA1, HMA2, and HMA3). The HMA1 protein is similar to the *Arabidopsis* HMA1, while HMA2 and HMA3 cluster with P_{1B}-type ATPases that are copper transporters, such as RAN1 in plants [121]. Although copper is an essential micronutrient, ion transport in this organism is not well understood. Recently, microarray analysis has been implemented to investigate gene transcription responses to external copper concentrations in Chlamydomonas. In addition to genes involved in metabolic processes, stress related and intracellular proteolysis genes were greatly affected by copper concentrations [122].

4.2. Copper Homeostasis in Higher Plants using *Arabidopsis* as a Model

Arabidopsis is a model organism for plant systems that is widely used in genetic and molecular investigations. Reasons for its use as a model include, a sequenced genome, short life cycle, and it is relatively easy to cross and mutate. Plants are complex, multicellular organisms that transport essential

micronutrients, like copper, over a long distance through organs and across several membranes before they reach their final destination in target proteins.

Copper enters through the cell membrane from the apoplast by a Ctr-like transporter called COPT1 [93], and is either sequestered or trafficked to targets by copper chaperones (Figure 7). The COPT1 protein and its four homologs are expressed in stems, flower, leaf, as well as root tissue [36] and *COPT1* antisense plants show abnormalities in pollen formation and increased root length [123]. This analysis suggests that COPT1 may play a role in copper uptake in the roots from surrounding environment. Little work has been done on the other proteins in the COPT family with localizations of proteins and molecular characterizations of mutants pending.

Arabidopsis contains two different cytosolic ATX-like copper chaperones, CCH and ATX1 [124,125]. Both proteins are functional homologs of the yeast ATX1 and have been shown to interact with the P-type ATPases, RAN1 in the Golgi membrane [125] and HMA5 in the plasma membrane [60]. The RAN1 (Responsive-to-antagonist1) transporter is a homolog of the yeast Ccc2 protein and functions to translocate copper into the endomembrane system where it is required for ethylene signaling [126,127]. Similar to RAN1, the HMA5 membrane transporter is highly expressed in roots and flowers, but based on mutant analysis the function seems to be copper detoxification in the roots [60].

Copper chaperones that are responsible for ion transport to the mitochondrion for cytochrome *c* oxidase are currently unknown. However, a homolog to the yeast Cox17 protein has been found in *Arabidopsis* that may

play a role in mitochondrial copper delivery [128]. *Arabidopsis* contains three Cu,Zn SOD enzymes located in the cytosol (CSD1), stroma (CSD2), and peroxisome (CSD3). Plants possess a homolog of the yeast CCS, also called CCS that is the copper chaperone for SOD. There is only one *CCS* gene in *Arabidopsis*; however it has been suggested that several start sites for transcription of the gene results in different subcellular locations [129,130].

Within *Arabidopsis* chloroplasts there are two P-type ATPases similar to the RAN1 transporter. The outer membrane of the chloroplast is porous and many ions, including copper perhaps, can diffuse readily through it. One of the P-type ATPases, PAA1, has been localized to the membrane (inner) of the chloroplast [131]. The second transporter, PAA2, has been localized using GFP (Green Fluorescent Protein) to the thylakoid membrane [132]. The PAA1 and PAA2 proteins are functional homologs of the CtaA and PacS, respectively, in cyanobacteria. Both of the PAA transporters contain a heavy metal binding motif, MxCxxC, at the N-terminal domain of the peptide along with the chloroplastic transit sequence. Although *paa1* mutants show a decrease in growth rate due to impairment of photosynthetic activity, it is not a lethal mutation [131]. As a result of this phenotype, it is thought that copper can enter the chloroplast membrane through an alternate route. Recently, another P-type ATPase, HMA1, has been localized to the chloroplastic membrane and it has been implicated as an alternate copper transporter for SOD activity [65].

In addition to the Cu,Zn SOD in the stroma, the chloroplast has a second more important target for copper, plastocyanin. Under varying copper

conditions, the plant must balance regulation of copper to both of these targets within the chloroplast. Obviously, photosynthetic activity will have a higher priority for acquiring copper, but the regulators for this homeostatic mechanism are unknown. Through GFP analysis, the CCS protein has been localized to the stroma [129] and additionally two other putative copper chaperones have been localized to the chloroplast [133,134]. The putative chaperone, CCP (Copper Chaperone for the Plastid), is somewhat similar to the bacterial CopZ and ATX1 proteins in sequence and has an ATX-like βαββαβ fold. However, CCP does not contain a typical metal binding domain (MxCxxC); instead it has a series of serine repeats [133]. The function of this protein is currently unknown; however one hypothesis is that it could be the chaperone for plastocyanin [133]. The second putative copper chaperone, CutA, also does not have a stereotypical metal binding site; however there is sequence similarity to a copper related protein in *E. coli* by the same name. This protein is expressed in all major plant tissues at similar levels and purified recombinant protein of the *Arabidopsis* CutA has been shown to bind Cu(II) at a level of nearly one mole copper per one mole of protein [134].

The chloroplast is a complex organelle and has several membranes for copper to transverse across. Even though two of the five target proteins for copper are located in the chloroplast, transport to the organelle and within are still not well understood. No copper chaperone has been identified for transport to PAA1 in the chloroplast membrane or to PAA2 in the thalykoid membrane for plastocyanin.

5. Objective of This Dissertation

Copper homeostasis in bacteria, yeast, cyanobacteria, plants, and even humans is a relatively conserved process. However, plants and cyanobacteria are unique because of their ability to photosynthesize. Since this process requires copper, a different system has evolved in plants to transport copper into and within the chloroplast membranes. The chloroplast is a unique organelle that contains several targets for copper and must communicate with the rest of the cell to acquire appropriate amounts of the metal for photosynthetic activity. The objective of this dissertation is to develop a better understanding of copper homeostasis in *Arabidopsis*. We hope to gain insight on the delivery pathways of copper in the plant cell by focusing on the proteins that are involved in transport.

References

[1] Wulfsberg, G. (2000) Inorganic chemistry, University Science Books. Sausalito, Calif.

[2] Linder, M.C. and Goode, C.A. (1991) Biochemistry of copper, Plenum Press. New York.

[3] Misra, K.C. (2000) Understanding mineral deposits, Kluwer Academic Publishers. Dordrecht ; Boston, Mass.

[4] Klein, C., Hurlbut, C.S., Dana, J.D. and Klein, C. (2002) The 22nd edition of the manual of mineral science : (after James D. Dana), J. Wiley. New York.

[5] Walker, J.C.G., Cornelis, K., Schidlowski, M., Schopf, J.W., Stevenson, J. and Walter, M.R. (1983) Environmental evolution of the Archean-Early Proterozoic Earth. In Earth's earliest biosphere: its origin and evolution (Schopf, J.W., ed.^eds), pp. 260-290. Princeton University Press, Princeton, NJ.

[6] Stevenson, J. (1983) The nature of the Earth prior to the oldest known rock record: the Hadean Earth. In Earth's earliest biosphere: its origin and evolution (Schopf, J.W., ed.^eds), pp. 32-40. Princeton University Press, Princeton, NJ.

[7] Chapman, D.J. and Schopf, J.W. (1983) Biological and biochemical effects of the development of an aerobic environment. In Earth's earliest biosphere: its origin and evolution (Schopf, J.W., ed.^eds), pp. 302-320. Princeton University Press, Princeton, NJ.

[8] Marschner, H. (1995) Mineral nutrition of higher plants, Academic Press. London ; San Diego.

[9] Hemphill, D.D. (1972). Availability of trace elements to plants with respect to soil-plant interaction. Annals New York Academy of Sciences 199, 46-61.

[10] Childers, N.F., Morris, J.R. and Sibbett, G.S. (1995) Modern fruit science: orchard and small fruit culture, Horticultural Publications. Gainesville, Fla.

[11] Pessarakli, M. (2005) Handbook of photosynthesis, Taylor & Francis. Boca Raton, FL.

[12] Bhattacharya, P.K. (2005) Metal ions in biochemistry, Alpha Science International Ltd. Harrow, U.K.

[13] Gavnholt, B. and Larsen, K. (2002). Molecular biology of plant laccases in relation to lignin formation. Physiologia Plantarum 116, 273-280.

[14] Horemans, N., Foyer, C.H. and Asard, H. (2000). Transport and action of ascorbate at the plant plasma membrane. Trends in Plant Science 5, 263-267.

[15] Smirnoff, N. (1996). The function and metabolism of ascorbic acid in plants. Annals of Botany 78, 661-669.

[16] Pignocchi, C., Fletcher, J.M., Wilkinson, J.E., Barnes, J.D. and Foyer, C.H. (2003). The Function of Ascorbate Oxidase in Tobacco. Plant Physiol 132, 1631-1641.

[17] Kato, N. and Esaka, M. (2000). Expansion of transgenic tobacco protoplasts expressing pumpkin ascorbate oxidase is more rapid than that of wild-type protoplasts. Planta 210, 1018-1022.
[18] Nakamura, K. and Go, N. (2005). Function and molecular evolution of multicopper blue proteins. Cell Mol Life Sci 62, 2050-66.
[19] Hulzink, R.J.M., de Groot, P.F.M., Croes, A.F., Quaedvlieg, W., Twell, D., Wullems, G.J. and van Herpen, M.M.A. (2002). The 5'-Untranslated Region of the ntp303 Gene Strongly Enhances Translation during Pollen Tube Growth, But Not during Pollen Maturation. Plant Physiol 129, 342-353.
[20] Sedbrook, J.C., Carroll, K.L., Hung, K.F., Masson, P.H. and Somerville, C.R. (2002). The Arabidopsis SKU5 Gene Encodes an Extracellular Glycosyl Phosphatidylinositol-Anchored Glycoprotein Involved in Directional Root Growth. Plant Physiol 14, 1635-1648.
[21] Michel, H., Behr, J., Harrenga, A. and Kannt, A. (1998). CYTOCHROME C OXIDASE: Structure and Spectroscopy. Annual Review of Biophysics and Biomolecular Structure 27, 329-356.
[22] Steffens, G.C.M., Biewald, R. and Buse, G. (1987). Cytochrome c oxidase is three-copper, two-heme-A protein. FEBS Journal 164, 295-300.
[23] Sigfridsson, K. (1998). Plastocyanin, an electron-transfer protein. Photosynthesis Research 57, 1-28.
[24] Bowler, C., Montagu, M.V. and Inze, D. (1992). Superoxide Dismutase and Stress Tolerance. Annual Review of Plant Physiology and Plant Molecular Biology 43, 83-116.
[25] Kliebenstein, D.J., Monde, R.A. and Last, R.L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118, 637-50.
[26] O'Halloran, T.V. and Culotta, V.C. (2000). Metallochaperones, an intracellular shuttle service for metal ions. J Biol Chem 275, 25057-60.
[27] Rae, T.D., Schmidt, P.J., Pufahl, R.A., Culotta, V.C. and V. O'Halloran, T. (1999) Undetectable Intracellular Free Copper: The Requirement of a Copper Chaperone for Superoxide Dismutaseed.^eds), pp. 805-808
[28] Puig, S., Lee, J., Lau, M. and Thiele, D.J. (2002). Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. J Biol Chem 277, 26021-30.
[29] Knight, S.A., Labbe, S., Kwon, L.F., Kosman, D.J. and Thiele, D.J. (1996). A widespread transposable element masks expression of a yeast copper transport gene. Genes Dev 10, 1917-29.
[30] Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J. and Klausner, R.D. (1994). Molecular characterization of a copper transport protein in S. cerevisiae: an unexpected role for copper in iron transport. Cell 76, 393-402.
[31] Georgatsou, E., Mavrogiannis, L.A., Fragiadakis, G.S. and Alexandraki, D. (1997). The yeast Fre1p/Fre2p cupric reductases facilitate copper

uptake and are regulated by the copper-modulated Mac1p activator. J Biol Chem 272, 13786-92.
[32] Hassett, R. and Kosman, D.J. (1995). Evidence for Cu(II) reduction as a component of copper uptake by Saccharomyces cerevisiae. J Biol Chem 270, 128-34.
[33] Harris, E.D. (2000). Cellular copper transport and metabolism. Annu Rev Nutr 20, 291-310.
[34] Puig, S. and Thiele, D.J. (2002). Molecular mechanisms of copper uptake and distribution. Curr Opin Chem Biol 6, 171-80.
[35] Dumay, Q.C., Debut, A.J., Mansour, N.M. and Saier, J.M.H. (2006). The Copper Transporter (Ctr) Family of Cu Uptake Systems. Journal of Molecular Microbiology & Biotechnology 11, 10-19.
[36] Sancenon, V., Puig, S., Mira, H., Thiele, D.J. and Penarrubia, L. (2003). Identification of a copper transporter family in Arabidopsis thaliana. Plant Mol Biol 51, 577-87.
[37] Kuhlbrandt, W. (2004). BIOLOGY, STRUCTURE AND MECHANISM OF P-TYPE ATPases. Nature Reviews Molecular Cell Biology 5, 282-295.
[38] Axelsen, K.B. and Palmgren, M.G. (2001). Inventory of the superfamily of P-type ion pumps in Arabidopsis. Plant Physiol 126, 696-706.
[39] Hall, J.L. and Williams, L.E. (2003). Transition metal transporters in plants. J Exp Bot 54, 2601-13.
[40] Williams, L.E., Pittman, J.K. and Hall, J.L. (2000). Emerging mechanisms for heavy metal transport in plants. Biochim Biophys Acta 1465, 104-26.
[41] Williams, L.E. and Mills, R.F. (2005). P1B-ATPases - an ancient family of transition metal pumps with diverse functions in plants. Trends in Plant Science 10, 491-502.
[42] Arguello, J.M., Eren, E. and Gonzalez-Guerrero, M. (2007). The structure and function of heavy metal transport P(1B)-ATPases. Biometals
[43] Rensing, C. and Grass, G. (2003). Escherichia coli mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev 27, 197-213.
[44] Pufahl, R.A., Singer, C.P., Peariso, K.L., Lin, S.J., Schmidt, P.J., Fahrni, C.J., Culotta, V.C., Penner-Hahn, J.E., and O'Halloran, T.V. (1997). Metal ion chaperone function of the soluble Cu(I) receptor Atx1. Science 278, 853-6.
[45] Harrison, M.D. and Dameron, C.T. (1999). Molecular mechanisms of copper metabolism and the role of the Menkes disease protein. J Biochem Mol Toxicol 13, 93-106.
[46] Rosenzweig, A.C. and O'Halloran, T.V. (2000). Structure and chemistry of the copper chaperone proteins. Current Opinion in Chemical Biology 4, 140-147.
[47] Rosenzweig, A.C., Huffman, D.L., Hou, M.Y., Wernimont, A.K., Pufahl, R.A. and O'Halloran, T.V. (1999). Crystal structure of the Atx1 metallochaperone protein at 1.02 A resolution. Structure 7, 605-17.

[48] Huffman, D.L. and O'Halloran, T.V. (2001). Function, structure, and mechanism of intracellular copper trafficking proteins. Annu Rev Biochem 70, 677-701.
[49] Culotta, V.C., Klomp, L.W., Strain, J., Casareno, R.L., Krems, B. and Gitlin, J.D. (1997). The copper chaperone for superoxide dismutase. J Biol Chem 272, 23469-72.
[50] Culotta, V.C., Yang, M. and O'Halloran, T.V. (2006). Activation of superoxide dismutases: putting the metal to the pedal. Biochim Biophys Acta 1763, 747-58.
[51] Lamb, A.L., Wernimont, A.K., Pufahl, R.A., O'Halloran, T.V. and Rosenzweig, A.C. (2000). Crystal structure of the second domain of the human copper chaperone for superoxide dismutase. Biochemistry 39, 1589-95.
[52] Lamb, A.L., Wernimont, A.K., Pufahl, R.A., Culotta, V.C., O'Halloran, T.V. and Rosenzweig, A.C. (1999). Crystal structure of the copper chaperone for superoxide dismutase. Nat Struct Biol 6, 724-9.
[53] Hall, L.T., Sanchez, R.J., Holloway, S.P., Zhu, H., Stine, J.E., Lyons, T.J., Demeler, B., Schirf, V., Hansen, J.C., Nersissian, A.M., Valentine, J.S., and Hart, P.J. (2000). X-ray crystallographic and analytical ultracentrifugation analyses of truncated and full-length yeast copper chaperones for SOD (LYS7): a dimer-dimer model of LYS7-SOD association and copper delivery. Biochemistry 39, 3611-23.
[54] Kagi, J.H., Kojima, Y., Kissling, M.M. and Lerch, K. (1979). Metallothionein: an exceptional metal thiolate protein. Ciba Found Symp, 223-37.
[55] Nielson, K.B. and Winge, D.R. (1984). Preferential binding of copper to the beta domain of metallothionein. J Biol Chem 259, 4941-6.
[56] Zhou, J. and Goldsbrough, P.B. (1994). Functional homologs of fungal metallothionein genes from Arabidopsis. Plant Cell 6, 875-84.
[57] Murphy, A. and Taiz, L. (1995). Comparison of metallothionein gene expression and nonprotein thiols in ten Arabidopsis ecotypes. Correlation with copper tolerance. Plant Physiol 109, 945-54.
[58] Guo, W.-J., Bundithya, W. and Goldsbrough, P.B. (2003). Characterization of the Arabidopsis metallothionein gene family: tissue-specific expression and induction during senescence and in response to copper. New Phytologist 159, 369-381.
[59] Arnesano, F., Banci, L., Bertini, I., Ciofi-Baffoni, S., Molteni, E., Huffman, D.L. and O'Halloran, T.V. (2002). Metallochaperones and metal-transporting ATPases: a comparative analysis of sequences and structures. Genome Res 12, 255-71.
[60] Andres-Colas, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker, J.R., Puig, S. and Penarrubia, L. (2006). The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J 45, 225-36.

[61] van Dongen, E.M., Klomp, L.W. and Merkx, M. (2004). Copper-dependent protein-protein interactions studied by yeast two-hybrid analysis. Biochem Biophys Res Commun 323, 789-95.
[62] Huffman, D.L. and O'Halloran, T.V. (2000). Energetics of copper trafficking between the Atx1 metallochaperone and the intracellular copper transporter, Ccc2. J Biol Chem 275, 18611-4.
[63] Tottey, S., Rondet, S.A., Borrelly, G.P., Robinson, P.J., Rich, P.R. and Robinson, N.J. (2002). A copper metallochaperone for photosynthesis and respiration reveals metal-specific targets, interaction with an importer, and alternative sites for copper acquisition. J Biol Chem 277, 5490-7.
[64] Axelsen, K.B. and Palmgren, M.G. (1998). Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol 46, 84-101.
[65] Seigneurin-Berny, D., Gravot, A., Auroy, P., Mazard, C., Kraut, A., Finazzi, G., Grunwald, D., Rappaport, F., Vavasseur, A., Joyard, J., Richaud, P., and Rolland, N. (2006). HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem 281, 2882-92.
[66] Rosenzweig, A.C. and O'Halloran, T.V. (2000). Structure and chemistry of the copper chaperone proteins. Curr Opin Chem Biol 4, 140-7.
[67] Gitschier, J., Moffat, B., Reilly, D., Wood, W.I. and Fairbrother, W.J. (1998). Solution structure of the fourth metal-binding domain from the Menkes copper-transporting ATPase. Nat Struct Biol 5, 47-54.
[68] Portnoy, M.E., Rosenzweig, A.C., Rae, T., Huffman, D.L., O'Halloran, T.V. and Culotta, V.C. (1999). Structure-function analyses of the ATX1 metallochaperone. J Biol Chem 274, 15041-5.
[69] Lu, Z.H., Dameron, C.T. and Solioz, M. (2003). The Enterococcus hirae paradigm of copper homeostasis: copper chaperone turnover, interactions, and transactions. Biometals 16, 137-43.
[70] Solioz, M. and Stoyanov, J.V. (2003). Copper homeostasis in Enterococcus hirae. FEMS Microbiol Rev 27, 183-95.
[71] Magnani, D. and Solioz, M. (2005). Copper chaperone cycling and degradation in the regulation of the cop operon of Enterococcus hirae. Biometals 18, 407-12.
[72] Odermatt, A., Suter, H., Krapf, R. and Solioz, M. (1993). Primary structure of two P-type ATPases involved in copper homeostasis in Enterococcus hirae. J Biol Chem 268, 12775-9.
[73] Odermatt, A., Krapf, R. and Solioz, M. (1994). Induction of the putative copper ATPases, CopA and CopB, of Enterococcus hirae by Ag+ and Cu2+, and Ag+ extrusion by CopB. Biochem Biophys Res Commun 202, 44-8.
[74] Odermatt, A. and Solioz, M. (1995). Two trans-acting metalloregulatory proteins controlling expression of the copper-ATPases of Enterococcus hirae. J Biol Chem 270, 4349-54.
[75] Strausak, D. and Solioz, M. (1997). CopY is a copper-inducible repressor of the Enterococcus hirae copper ATPases. J Biol Chem 272, 8932-6.

[76] Cobine, P., Wickramasinghe, W.A., Harrison, M.D., Weber, T., Solioz, M. and Dameron, C.T. (1999). The Enterococcus hirae copper chaperone CopZ delivers copper(I) to the CopY repressor. FEBS Lett 445, 27-30.
[77] Wimmer, R., Herrmann, T., Solioz, M. and Wuthrich, K. (1999). NMR structure and metal interactions of the CopZ copper chaperone. J Biol Chem 274, 22597-603.
[78] Benov, L.T. and Fridovich, I. (1994). Escherichia coli expresses a copper- and zinc-containing superoxide dismutase. J Biol Chem 269, 25310-4.
[79] Gort, A.S., Ferber, D.M. and Imlay, J.A. (1999). The regulation and role of the periplasmic copper, zinc superoxide dismutase of Escherichia coli. Mol Microbiol 32, 179-91.
[80] Osborne, J.P., Cosper, N.J., Stalhandske, C.M., Scott, R.A., Alben, J.O. and Gennis, R.B. (1999). Cu XAS shows a change in the ligation of CuB upon reduction of cytochrome bo3 from Escherichia coli. Biochemistry 38, 4526-32.
[81] Rapisarda, V.A., Montelongo, L.R., Farias, R.N. and Massa, E.M. (1999). Characterization of an NADH-linked cupric reductase activity from the Escherichia coli respiratory chain. Arch Biochem Biophys 370, 143-50.
[82] Rapisarda, V.A., Chehin, R.N., De Las Rivas, J., Rodriguez-Montelongo, L., Farias, R.N. and Massa, E.M. (2002). Evidence for Cu(I)-thiolate ligation and prediction of a putative copper-binding site in the Escherichia coli NADH dehydrogenase-2. Arch Biochem Biophys 405, 87-94.
[83] Beswick, P.H., Hall, G.H., Hook, A.J., Little, K., McBrien, D.C. and Lott, K.A. (1976). Copper toxicity: evidence for the conversion of cupric to cuprous copper in vivo under anaerobic conditions. Chem Biol Interact 14, 347-56.
[84] Rensing, C., Fan, B., Sharma, R., Mitra, B. and Rosen, B.P. (2000). CopA: An Escherichia coli Cu(I)-translocating P-type ATPase. Proc Natl Acad Sci U S A 97, 652-6.
[85] Grass, G. and Rensing, C. (2001). CueO is a multi-copper oxidase that confers copper tolerance in Escherichia coli. Biochem Biophys Res Commun 286, 902-8.
[86] Stoyanov, J.V., Hobman, J.L. and Brown, N.L. (2001). CueR (Ybbl) of Escherichia coli is a MerR family regulator controlling expression of the copper exporter CopA. Mol Microbiol 39, 502-11.
[87] Outten, F.W., Outten, C.E., Hale, J. and O'Halloran, T.V. (2000). Transcriptional activation of an Escherichia coli copper efflux regulon by the chromosomal MerR homologue, cueR. J Biol Chem 275, 31024-9.
[88] Munson, G.P., Lam, D.L., Outten, F.W. and O'Halloran, T.V. (2000). Identification of a copper-responsive two-component system on the chromosome of Escherichia coli K-12. J Bacteriol 182, 5864-71.

[89] Franke, S., Grass, G., Rensing, C. and Nies, D.H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of Escherichia coli. J Bacteriol 185, 3804-12.
[90] Labbe, S., Zhu, Z. and Thiele, D.J. (1997). Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. J Biol Chem 272, 15951-8.
[91] Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D.J., Klausner, R.D. and Dancis, A. (1997). Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. J Biol Chem 272, 17711-8.
[92] Zhu, Z., Labbe, S., Pena, M.M. and Thiele, D.J. (1998). Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. J Biol Chem 273, 1277-80.
[93] Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D. and Van Montagu, M. (1995). Molecular characterization of a putative Arabidopsis thaliana copper transporter and its yeast homologue. J Biol Chem 270, 28479-86.
[94] Portnoy, M.E., Schmidt, P.J., Rogers, R.S. and Culotta, V.C. (2001). Metal transporters that contribute copper to metallochaperones in Saccharomyces cerevisiae. Mol Genet Genomics 265, 873-82.
[95] Rees, E.M., Lee, J. and Thiele, D.J. (2004). Mobilization of intracellular copper stores by the ctr2 vacuolar copper transporter. J Biol Chem 279, 54221-9.
[96] Yamaguchi, Y., Heiny, M.E., Suzuki, M. and Gitlin, J.D. (1996). Biochemical characterization and intracellular localization of the Menkes disease protein. Proc Natl Acad Sci U S A 93, 14030-5.
[97] Lin, S.J., Pufahl, R.A., Dancis, A., O'Halloran, T.V. and Culotta, V.C. (1997). A role for the Saccharomyces cerevisiae ATX1 gene in copper trafficking and iron transport. J Biol Chem 272, 9215-20.
[98] Yuan, D.S., Dancis, A. and Klausner, R.D. (1997). Restriction of copper export in Saccharomyces cerevisiae to a late Golgi or post-Golgi compartment in the secretory pathway. J Biol Chem 272, 25787-93.
[99] Askwith, C.C., de Silva, D. and Kaplan, J. (1996). Molecular biology of iron acquisition in Saccharomyces cerevisiae. Mol Microbiol 20, 27-34.
[100] Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T. and Klausner, R.D. (1995). The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. Proc Natl Acad Sci U S A 92, 2632-6.
[101] De Silva, D.M., Askwith, C.C., Eide, D. and Kaplan, J. (1995). The FET3 gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. J Biol Chem 270, 1098-101.
[102] Beers, J., Glerum, D.M. and Tzagoloff, A. (1997). Purification, characterization, and localization of yeast Cox17p, a mitochondrial copper shuttle. J Biol Chem 272, 33191-6.
[103] Nobrega, M.P., Bandeira, S.C., Beers, J. and Tzagoloff, A. (2002). Characterization of COX19, a widely distributed gene required for

expression of mitochondrial cytochrome oxidase. J Biol Chem 277, 40206-11.
[104] Cobine, P.A., Pierrel, F., Bestwick, M.L. and Winge, D.R. (2006). Mitochondrial matrix copper complex used in metallation of cytochrome oxidase and superoxide dismutase. J Biol Chem 281, 36552-9.
[105] Horng, Y.C., Cobine, P.A., Maxfield, A.B., Carr, H.S. and Winge, D.R. (2004). Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome C oxidase. J Biol Chem 279, 35334-40.
[106] Balatri, E., Banci, L., Bertini, I., Cantini, F. and Ciofi-Baffoni, S. (2003). Solution structure of Sco1: a thioredoxin-like protein Involved in cytochrome c oxidase assembly. Structure 11, 1431-43.
[107] Hiser, L., Di Valentin, M., Hamer, A.G. and Hosler, J.P. (2000). Cox11p is required for stable formation of the Cu(B) and magnesium centers of cytochrome c oxidase. J Biol Chem 275, 619-23.
[108] Schmidt, P.J., Ramos-Gomez, M. and Culotta, V.C. (1999). A gain of superoxide dismutase (SOD) activity obtained with CCS, the copper metallochaperone for SOD1. J Biol Chem 274, 36952-6.
[109] Schmidt, P.J., Rae, T.D., Pufahl, R.A., Hamma, T., Strain, J., O'Halloran, T.V. and Culotta, V.C. (1999). Multiple protein domains contribute to the action of the copper chaperone for superoxide dismutase. J Biol Chem 274, 23719-25.
[110] Sturtz, L.A., Diekert, K., Jensen, L.T., Lill, R. and Culotta, V.C. (2001). A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. J Biol Chem 276, 38084-9.
[111] Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J. and Fogel, S. (1984). Primary structure and transcription of an amplified genetic locus: the CUP1 locus of yeast. Proc Natl Acad Sci U S A 81, 337-41.
[112] Culotta, V.C., Howard, W.R. and Liu, X.F. (1994). CRS5 encodes a metallothionein-like protein in Saccharomyces cerevisiae. J Biol Chem 269, 25295-302.
[113] Thiele, D.J. (1988). ACE1 regulates expression of the Saccharomyces cerevisiae metallothionein gene. Mol Cell Biol 8, 2745-52.
[114] Gralla, E.B., Thiele, D.J., Silar, P. and Valentine, J.S. (1991). ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. Proc Natl Acad Sci U S A 88, 8558-62.
[115] Kerfield, C.A. and Krogmann, D.W. (1998). Photosynthetic cytochromes c in cyanobacteria, algae, and plants. Annual Review of Plant Physiology and Plant Molecular Biology 49, 397-425.
[116] Dworsky, A., Mayer, B., Regelsberger, G., Fromwald, S. and Peschek, G.A. (1995). Functional and immunological characterization of both "mitochondria-like" and "chloroplast-like" electron/proton transport

proteins in isolated and purified cyanobacterial membranes. Bioelectrochemistry and Bioenergetics 38, 35-43.
[117] Zhang, L., McSpadden, B., Pakrasi, H.B. and Whitmarsh, J. (1992). Copper-mediated regulation of cytochrome c553 and plastocyanin in the cyanobacterium Synechocystis 6803. J Biol Chem 267, 19054-9.
[118] Phung, L.T., Ajlani, G. and Haselkorn, R. (1994). P-type ATPase from the cyanobacterium Synechococcus 7942 related to the human Menkes and Wilson disease gene products. Proc Natl Acad Sci U S A 91, 9651-4.
[119] Kanamaru, K., Kashiwagi, S. and Mizuno, T. (1994). A copper-transporting P-type ATPase found in the thylakoid membrane of the cyanobacterium Synechococcus species PCC7942. Mol Microbiol 13, 369-77.
[120] Merchant, S.S., Allen, M.D., Kropat, J., Moseley, J.L., Long, J.C., Tottey, S. and Terauchi, A.M. (2006). Between a rock and a hard place: trace element nutrition in Chlamydomonas. Biochim Biophys Acta 1763, 578-94.
[121] Hanikenne, M., Kramer, U., Demoulin, V. and Baurain, D. (2005). A comparative inventory of metal transporters in the green alga Chlamydomonas reinhardtii and the red alga Cyanidioschizon merolae. Plant Physiol 137, 428-46.
[122] Jamers, A., Van der Ven, K., Moens, L., Robbens, J., Potters, G., Guisez, Y., Blust, R. and De Coen, W. (2006). Effect of copper exposure on gene expression profiles in Chlamydomonas reinhardtii based on microarray analysis. Aquat Toxicol 80, 249-60.
[123] Sancenon, V., Puig, S., Mateu-Andres, I., Dorcey, E., Thiele, D.J. and Penarrubia, L. (2004). The Arabidopsis copper transporter COPT1 functions in root elongation and pollen development. J Biol Chem 279, 15348-55.
[124] Himelblau, E., Mira, H., Lin, S.J., Culotta, V.C., Penarrubia, L. and Amasino, R.M. (1998). Identification of a functional homolog of the yeast copper homeostasis gene ATX1 from Arabidopsis. Plant Physiol 117, 1227-34.
[125] Puig, S., Mira, H., Dorcey, E., Sancenon, V., Andres-Colas, N., Garcia-Molina, A., Burkhead, J.L., Gogolin, K.A., Abdel-Ghany, S.E., Thiele, D.J., Ecker, J.R., Pilon, M., Penarrubia, L. (2007). Higher plants possess two different types of ATX1-like copper chaperones. Biochemical and Biophysical Research Communications 354, 385-390.
[126] Hirayama, T. and Alonso, J.M. (2000). Ethylene captures a metal! Metal ions are involved in ethylene perception and signal transduction. Plant Cell Physiol 41, 548-55.
[127] Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker J.R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97, 383-93.

[128] Balandin, T. and Castresana, C. (2002). AtCOX17, an Arabidopsis homolog of the yeast copper chaperone COX17. Plant Physiol 129, 1852-7.
[129] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., Andres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Letters 579, 2307-2312.
[130] Chu, C.C., Lee, W.C., Guo, W.Y., Pan, S.M., Chen, L.J., Li, H.M. and Jinn, T.L. (2005). A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. Plant Physiol 139, 425-36.
[131] Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K.K. and Pilon, M. (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15, 1333-46.
[132] Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M. and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17, 1233-51.
[133] Burkhead, J. and Colorado State University. Dept. of Biology. (2003) Copper traffic in plants : roles for newly isolated chloroplast proteins, pp. 145. Colorado State University, Fort Collins, CO.
[134] Burkhead, J.L., Abdel-Ghany, S.E., Morrill, J.M., Pilon-Smits, E.A. and Pilon, M. (2003). The Arabidopsis thaliana CUTA gene encodes an evolutionarily conserved copper binding chloroplast protein. Plant J 34, 856-67.
[135] Peitsch, M.C. (1995). Protein modeling by E-mail. Bio/Technology 13, 658-660.
[136] Guex, N. and Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714-23.
[137] Schwede, T., Kopp, J., Guex, N. and Peitsch, M.C. (2003). SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 31, 3381-5.
[138] Nittis, T., George, G.N. and Winge, D.R. (2001). Yeast Sco1, a protein essential for cytochrome c oxidase function is a Cu(I)-binding protein. J Biol Chem 276, 42520-6.
[139] Winge, D.R. (2003). Let's Sco1, Oxidase! Let's Sco! Structure 11, 1313-4.
[140] Beers, J., Glerum, D.M. and Tzagoloff, A. (2002). Purification and characterization of yeast Sco1p, a mitochondrial copper protein. J Biol Chem 277, 22185-90.
[141] Katoh, H., Hagino, N. and Ogawa, T. (2001). Iron-binding activity of FutA1 subunit of an ABC-type iron transporter in the cyanobacterium Synechocystis sp. Strain PCC 6803. Plant Cell Physiol 42, 823-7.
[142] Waldron, K.J., Tottey, S., Yanagisawa, S., Dennison, C. and Robinson, N.J. (2007). A periplasmic iron-binding protein contributes toward inward copper supply. J Biol Chem 282, 3837-46.

[143] Katoh, H., Hagino, N., Grossman, A.R. and Ogawa, T. (2001). Genes essential to iron transport in the cyanobacterium Synechocystis sp. strain PCC 6803. J Bacteriol 183, 2779-84.
[144] Bowler, C. (1992). Superoxide Dismutase and Stress Tolerance. Annual Review of Plant Physiology and Plant Molecular Biology 43, 83.

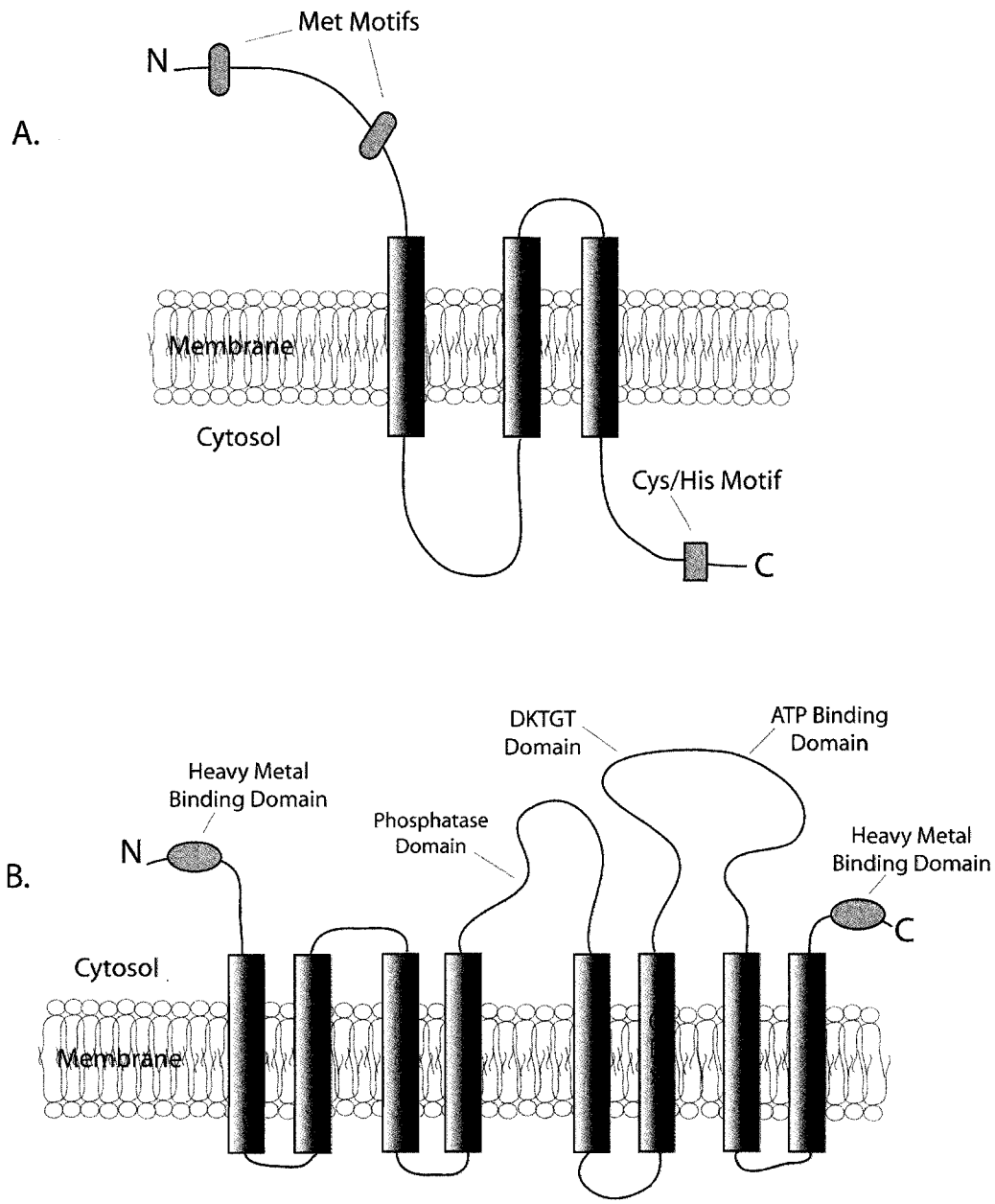

Figure 1. Schematic diagrams showing the Ctr Family of transporters in yeast (Panel A) and the Heavy Metal Associated (HMA) Family of P_{1B}-type ATPase transporters in *Arabidopsis* (Panel B)

Figure 2. A ribbon model of an ATX-like copper chaperone depicting a typical βαββαβ (ferredoxin-like) fold. Model was made using SWISS-MODEL and DeepView/Swiss-PbdViewer software [135-137].

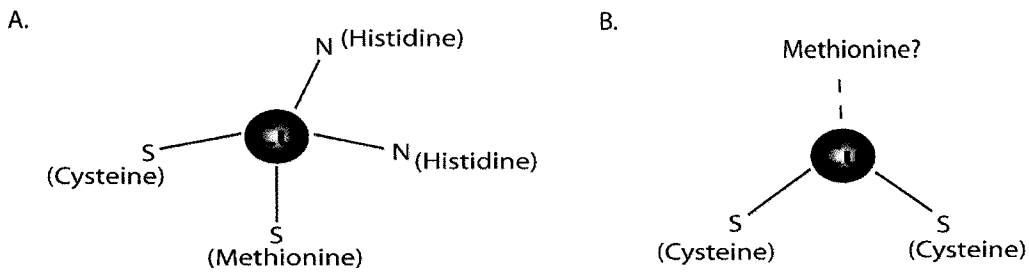

Figure 3. Diagrams depicting copper coordinating sites in the blue copper binding protein, plastocyanin (A.) and possible copper binding sites in copper transporters and chaperones containing an MxCxxC heavy metal domain (B.).

Table 1. A list of copper trafficking proteins in bacterial models.

Copper Transport Related Proteins and Regulatory Elements	Cellular location	Function	Homologs	References
Bacteria				
Enterococcus hirae				
Reductase?	Extracellular	Reduces Cu(II) to Cu(I) for uptake	Ndh-2 *(E. coli)*	
P-type ATPase Transporters				
CopA	Plasma membrane	Cu(I) influx transporter		[72,73]
CopB	Plasma membrane	Cu(I) efflux transporter	CopA *(E. coli)*	[72,73]
Cu Metallochaperone				
CopZ	Cytoplasm	Delivers Cu(I) to CopY		[74,76]
Transcriptional Regulation				
CopY	Cytoplasm	Copper-responsive repressor		[74,75]
Escherichia coli				
Reductase				
NDH-2	Inner membrane	Reduces Cu(II) to Cu(I) for uptake		[81,82]
P-type ATPase Transporters				
CopA	Inner membrane	Cu(I) efflux transporter	CopB *(E. hirae)*	[84]
Four-component Copper Pump				
CusCFBA	Inner membrane, periplasm, outer membrane	Cu(I) efflux		[88,89]
Multi-copper oxidase				
CueO	Periplasm	Protection of the periplasm from copper-induced damage		[85]
Targets for Cu(I)				
Cu,Zn SOD	Periplasm	Enzyme that reduces reactive oxidative stress		
Cytochrome bo3	Periplasm	Laccase activity, detoxification?		[80]
Transcriptional Regulation				
CueR	Cytoplasm	DNA-binding regulator; member of the MerR family of metal-responsive regulators		[86,87]
CusRS	Cytoplasm	Chromosomal two-component regulator		[88]

Table 2. A list of copper trafficking proteins in yeast.

Copper Transport Related Proteins and Regulatory Elements	Cellular location	Function	Homologs	References
Yeast				
Sacchaomyces cerevisae				
Reductases				
Fre1/Fre2	Extracellular	Reduces Cu(II) to Cu(I) for uptake		[31,32]
Copper Transporters (Ctr)				
Ctr1	Plasma membrane	High affinity Cu(I) influx	COPT1 *(A. thaliana)*	[30]
Ctr2	Vacuole membrane?	Releases stores of copper from vacuole		[93-95]
Ctr3	Plasma membrane	High affinity Cu(I) influx		[29]
P-type ATPase Transporters				
Ccc2	Golgi membrane	Uptake of copper from cytosol to endomembrane system for incorporation into Fet3 for iron uptake	RAN1*(A. thaliana)*	[96]
Copper Chaperones				
ATX1	Ctyoplasm	Interacts with Ccc2	Atx1 *(Synechocystis)* Atx1 *(A. thaliana)* CCH *(A. thaliana)*	[97]
CCS	Cytoplasm	Transports Cu(I) from cell membrane to SOD1 (Cu,Zn superoxide dismutase)	CCS *(A. thaliana)*	[108,109]
Cox17 and Cox19	Mitochondrion	Incorporation of copper into cytochrome *c* oxidase in mitochondrion	Cox17 *(A. thaliana)*	[102,103]
Cox11	Mitochondrion	Incorporation of copper into cytochrome c oxidase		[105,107]
Sco1	Mitochondrion	Incorporation of copper into cytochrome c oxidase		[138-140]
Targets for Cu(I)				
Fet3	Extracellular	Multi-copper ferroxidase/iron permease; high affinity iron uptake		[30,99-101]
SOD1	Cytoplasm	Enzyme that reduces reactive oxidative stress	CSD1 *(A. thaliana)*	
Cco	Mitochondrion	Terminal electron acceptor in respiration		
Transcriptional Regulation				
Mac1	Nucleus	Transcriptional activation of *FRE1, CTR1, CTR3*		[31,90-92]
Ace1	Nucleus	Transcriptional activation of *SOD1, CRS5, CUP1*		[112-114]
Metallothionein				
Crs5	Cytoplasm	Sequesters excess copper		[112]
Cup1	Cytoplasm	Sequesters excess copper		[111]

Table 3. A list of copper trafficking proteins in photosynthetic cyanobacteria.

Copper Transport Related Proteins	Cellular location	Function	Homologs	References
Cyanobacteria				
Synechocystis				
Copper Import				
FutA1	Periplasm	Aids in Cu import		[141,142]
FutA2	Periplasm	Aids in Cu import		[142,143]
P-type ATPase Transporters				
CtaA	Plasma membrane	Cu(I) influx	PAA1 *(A. thaliana)*	[118]
PacS	Thylakoid membrane	Cu(I) influx	PAA2 *(A. thaliana)*	[119]
Cu Metallochaperones				
Atx1	Cytoplasm	Transports Cu(I) from cell membrane to thylakoid membrane	Atx1 *(S. cerevisae)* Atx1 *(A. thaliana)* CCH *(A. thaliana)*	[63]
Targets for Cu(I)				
PC (Plastocyanin)	Thylakoid	Electron transport in photosynthesis		
CO (Cytochrome oxidase)	Thylakoid	Electron transport in respiration		

Table 4. A list of copper trafficking proteins in plants.

Copper Transport Related Proteins	Cellular location	Function	Homologs	References
Plants				
Arabidopsis thaliana				
Copper Transporters (Ctr-like)				
COPT1	Cell membrane	Cu(I) influx	Ctr1 *(S. cerevisae)*	[36,93,123]
COPT2	Unknown	Unknown		[36]
COPT3	Unknown	Unknown		[36]
COPT4	Unknown	Unknown		[36]
COPT5	Unknown	Unknown		[36]
P-type ATPase Transporters				[36]
PAA1	Chloroplast membrane	Cu(I) influx into the choloplast	CtaA *(Synechocystis)*	[131]
HMA1	Chloroplast membrane	Secondary copper importer?		[65]
PAA2	Thylakoid membrane	Cu(I) influx into the thylakoid for plastocyanin	PacS *(Synechocystis)*	[132]
RAN1	Golgi membrane	Cu(I) influx into Golgi complex for ethylene response	Ccc2 *(S. cerevisae)*	[60,127]
HMA5	Plasma membrane	Copper detoxification		[60]
Cu Metallochaperones				
COX17	Mitochondrion		COX17 *(S. cerevisae)*	[128]
CCH	Cytoplasm	Interacts with RAN1 and HMA5	Atx1 *(S. cerevisae)* Atx1 *(Synechocystis)*	[60,124]
ATX1	Cytoplasm	Interacts with RAN1 and HMA5	Atx1 *(S. cerevisae)* Atx1 *(Synechocystis)*	[124,125]
CCP	Chloroplast	Function unknown		[133]
CCS	Chloroplast and Cytoplasm	Transports Cu(I) to CSD2 and CSD1?	CCS *(S. cerevisae)*	[129,130]
CutA	Chloroplast Envelope?	Unknown	CUTA *(E. coli.)*	[134]
Targets for Cu(I)				
ETR	Endomembrane System	Ethylene signaling response		[126,127]
Cyt-*c* (Cytochrome *c* oxidase)	Mitochondrion	Electron transport in respiration		
PC (Plastocyanin)	Thylakoid	Electron transport in photosynthesis		
CSD1(Cu,Zn SOD)	Cytoplasm	Enzyme that reduces reactive oxidative stress		[25,144]
CSD2 (Cu,Zn SOD)	Chloroplast	Enzyme that reduces reactive oxidative stress		[25,144]
CSD3 (Cu,Zn SOD)	Peroxisome	Enzyme that reduces reactive oxidative stress		[25,144]
Laccases				
Ascorbate Oxidase				
Polyphenol Oxidase				
Plantacyanin				

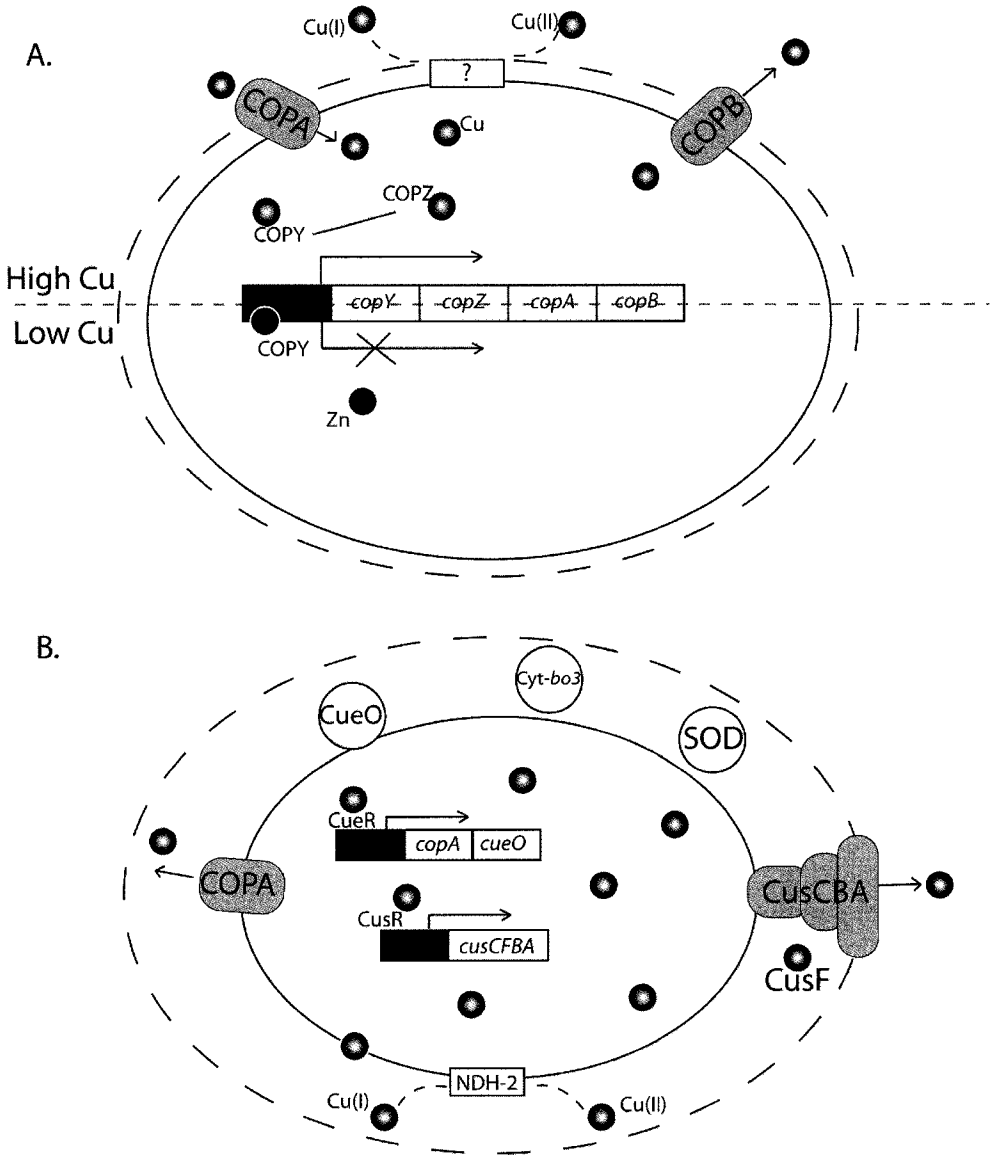

Figure 4. Illustrations of copper homeostasis in two bacterial models, *E. hirea* (Panel A) and *E. coli* (Panel B) (white rectangles symbolize copper reductases, gray rectangles are copper transporters, and white circles represent copper targets).

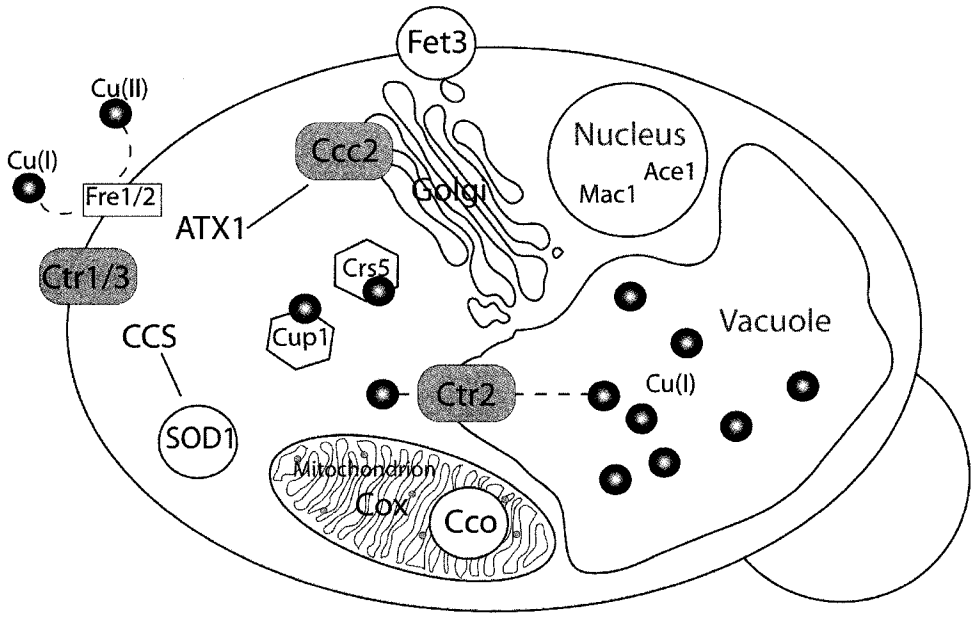

Figure 5. Copper trafficking proteins and pathways in *Sacchaomyces cerevisae* (solid line depicts interactions demonstrated through experimental data, white rectangles symbolize copper reductases, gray rectangles are copper transporters, white circles represent copper targets, and white hexagons are metallothioneins).

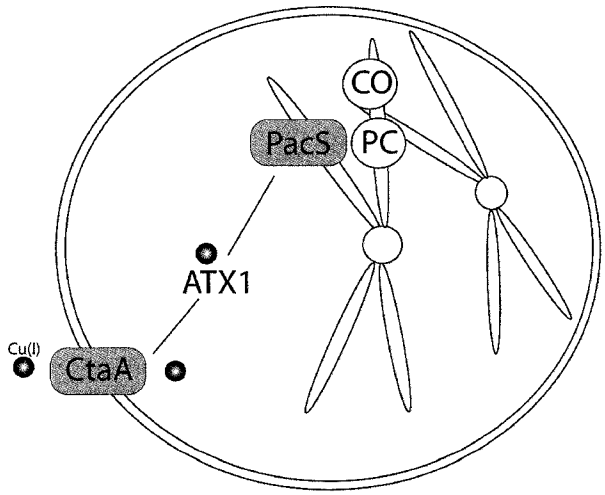

Figure 6. Copper homeostasis in the cyanobacterial model, *Synechocystis* (solid line depicts interactions demonstrated through experimental data, gray rectangles are copper transporters, and white circles represent copper targets).

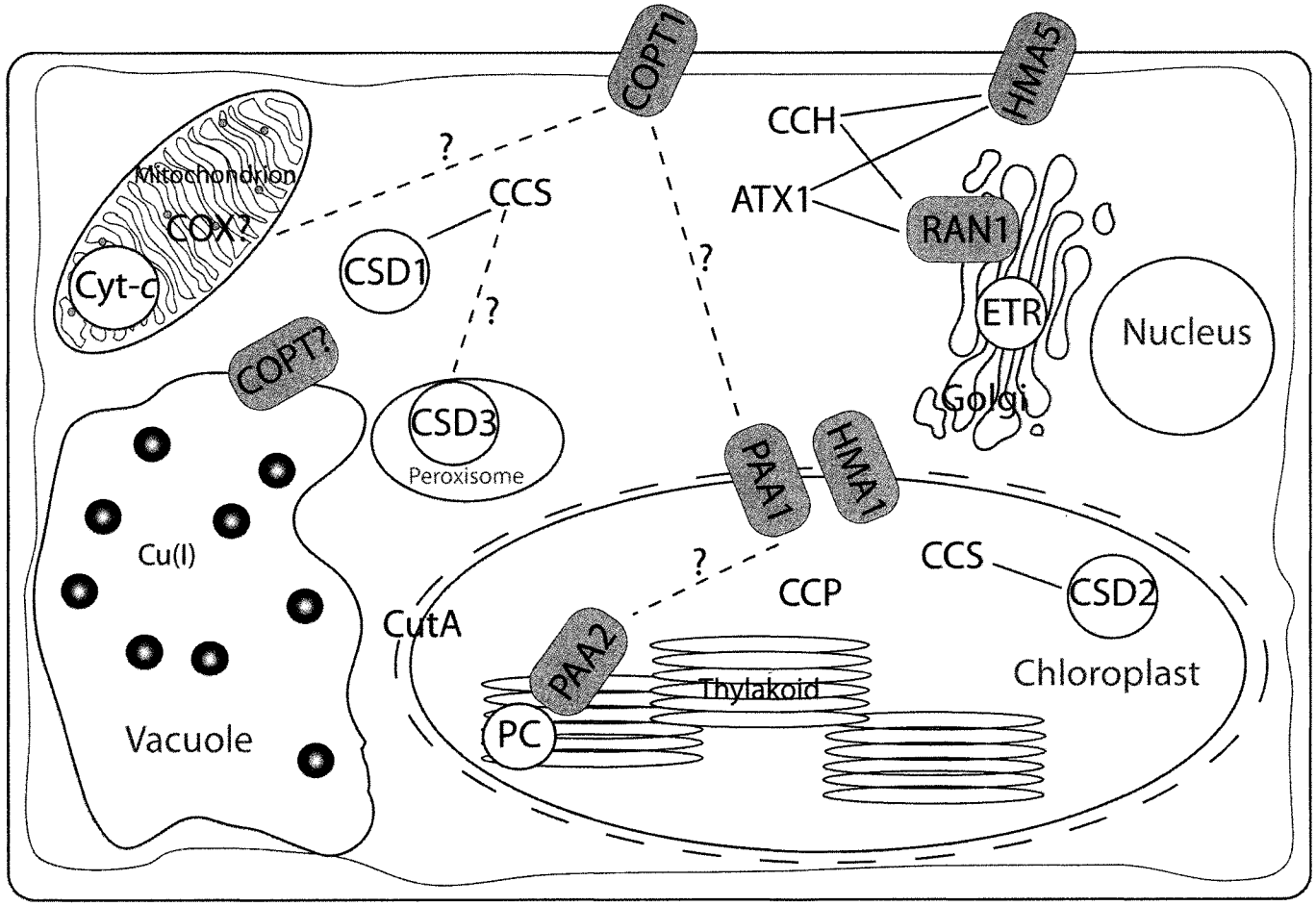

Figure 7. Copper transport pathways in *Arabidopsis thaliana* (solid lines depict interactions demonstrated through experimental data, dashed lines are hypothetical pathways, gray rectangles are copper transporters, and white circles represent copper targets

CHAPTER 2

Further characterization of PAA1 and PAA2 in *Arabidopsis* and a new model for copper transport in the chloroplast

Abstract

Plastocyanin is a small, blue-copper containing protein that is found in the thylakoid lumen of chloroplasts and it is one of the most abundant proteins found in photosynthetic tissue. Unlike some cyanobacteria that are able to use an iron containing protein for transport of electrons *Arabidopsis* requires plastocyanin for photosynthetic activity. Several P_{1B}-type ATPase transporters have been identified in *Arabidopsis.* The PAA1 and PAA2 transporters are functional homologs of the cyanobacterial CtaA and PacS and have been localized to the chloroplast envelope and thylakoid membranes, respectively. The *paa1* and *paa2* mutants have previously been characterized and display a decreased growth rate, a high chlorophyll fluorescence phenotype, as well as a decrease accumulation of plastocyanin protein. In this investigation, we further characterize the *paa1-1*, *paa1-3*, and *paa2-1* mutant plants and determined that the mutations affect copper homeostasis differently in root and shoot tissue. We also observe a distinct difference in chloroplast structure between mutant plants and wildtype controls. Additionally, we provide

evidence that P_{1B}-type ATPase transporters can interact with each other via ATX-like domains. This novel interaction between PAA1(N) and PAA2(N) has led us to a new model for copper transport in the chloroplast.

Abbreviations used:

CCS, copper chaperone for Cu/Zn superoxide dismutase; Cu,ZnSOD, Cu,Zn superoxide dismutase; CSD1, cytosolic Cu,Zn SOD; CSD2, stromal Cu,Zn SOD; FeSOD, Fe superoxide dismutase; MnSOD, Mn superoxide dismutase; PC, plastocyanin; SOD, superoxide dismutase.

1. Introduction

Plants are sessile organisms that obtain all essential components for life from the immediate environment in which they live. In this environment, plants must regulate the uptake of micronutrients to ensure that requirements are met for the important biological processes photosynthesis and respiration. Furthermore, these nutrients must travel long distances to reach aboveground organs and across several membranes before they are incorporated into target enzymes. Copper is one micronutrient that is required for both photosynthesis and respiration in higher plants.

Plastocyanin is a small (10 kDa), blue-copper containing protein that is found in the thylakoid lumen of chloroplasts and it is one of the most abundant proteins found in photosynthetic tissue. There are two plastocyanin genes that encode similar proteins in *Arabidopsis thaliana* [1]. Some cyanobacteria can use the iron containing cytochrome c_6 protein for transport of electrons

between the b/f complex and photosystem I in photosynthesis instead of the copper-requiring protein plastocyanin [2]. However, higher plants such as *Arabidopsis* do not have an alternative and require plastocyanin for photosynthetic activity.

Copper enters the plant cell through the copper transporter, COPT1 [3], and gets shuttled to one of its many targets via copper chaperones. These targets include, plastocyanin, cytochrome *c* oxidase, an ethylene receptor, and several Cu,Zn superoxide dismutase enzymes. *Arabidopsis* contains three isoforms of Cu,ZnSODs which are located in the cytosol, peroxisome, and the chloroplast [4]. In addition, there are eight different Heavy Metal Associated (HMA) transporters, several of which have been identified as copper transporters. The HMA5 membrane transporter is highly expressed in roots and flowers, however based on mutant analysis the function seems to be detoxification of copper within root tissue [5]. Responsive-to-antagonist1 (RAN1) is located in the Golgi membrane and delivers copper to an ethylene response protein within the endomembrane system [6]. The transporters, PAA1 and PAA2, have been localized to the chloroplast envelope and thylakoid membranes, respectively [7,8]. The PAA1 and PAA2 transporters contain an MxCxxC copper binding motif and are functional homologs of the cyanobacterial CtaA and PacS, respectively.

PAA1 and *PAA2* are both expressed in the shoots, however only *PAA1* is expressed in the root tissue. The *paa1* and *paa2* mutants display a decreased growth rate in which *paa1* is more severe than *paa2*. Both mutants also exhibit a high chlorophyll fluorescence phenotype that suggests a defect

in the electron transport pathway. Immunoblotting analysis has shown that *paa1* and *paa2* have a decreased accumulation of plastocyanin which most likely is the cause of the growth rate and fluorescence phenotypes. Interestingly, these phenotypes can be overcome by copper feeding [7,8].

Even though copper is an essential micronutrient, due to its redox potential it is also extremely toxic to the cell. Therefore, plants have adapted a mechanism to decrease the amount free copper ions through the presence of many proteins involved in copper transport and sequestration. Most plants contain 5 - 20 μg/g copper (dry weight) and *Arabidopsis* exhibits visible signs of toxicity with media concentrations as low as 20 μM [9,10]. Plastocyanin is arguable one of the most important targets for copper; however it is not the only target for copper within the chloroplast.

The chloroplast is a complex organelle and has several membranes for copper to transverse across. Even though the important copper requiring proteins plastocyanin and CSD2 are located in the chloroplast little is known about the transport pathway for this essential metal. Currently, there has been no copper chaperone identified for the transport of the ion to PAA1 for import into the chloroplast or one that is responsible for transport to PAA2 at the thylakoid membrane for plastocyanin.

In this investigation we further characterize the *paa1* and *paa2* mutants by studying the regulation of copper homeostasis in various copper treatments and we note distinct differences in chloroplast ultrastructure caused by a mutation in the genes. Finally, we propose a new possible model for copper

transport from PAA1 to PAA2 in the chloroplast through evidence of a protein-protein interaction detected by yeast two-hybrid.

2. Materials and Methods

Plant material and growth conditions. Arabidopsis ecotype Colombia-O was used as a wildtype. The *paa1-1*, *paa1-3*, and *paa2-1* mutants have been described [7,8]. *Arabidopsis* plants were grown on one half Murashige and Skoog (MS) medium including 1% sucrose and 0.4% agar gel [11]. The medium was supplemented with $CuSO_4$ (Sigma # C9012; Sigma, St Louis) as indicated.

Protein Analysis by Immunoblotting and Native Assays. Soluble protein extraction of plant tissue for SDS-PAGE and NATIVE-PAGE analysis was performed according to [12]. Protein was quantified using the Bradford method [13] and bovine serum albumin was used for a standard curve. A total of 10 µg of protein extract was fractionated on PAGE gels for SOD activity analysis or immunodetection. Activity of SODs was determined by staining NATIVE-PAGE gels according to [8,14] and 10-20% Tris-HCL gradient gels (Bio-Rad Laboratories) were used to resolve CSD1 and CSD2 activity. Antibodies used for immunodetection were obtained from D. Kliebenstein (University of California, Davis, CA) [4] or have been described [8] with the exception of CCS. The amino terminal domains of PAA1 and PAA2 as well as the full length CCS recombinant proteins were expressed in *E. coli* and purified using affinity chromatography. Purified proteins were then used to produce polyclonal

antibodies in rabbits (Pocono Rabbit Farm and Laboratory Inc, PA, USA). All antibodies were raised against *Arabidopsis* purified proteins with the exception of plastocyanin which was raised against spinach.

Transmission Electron Microscopy. Vegetative tissue from 14 day old plants were fixed in 5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 2 hours, then rinsed three times in 0.1M sodium cacodylate buffer, and post-fixed in 2% osmium tetroxide in 0.1M sodium cacodylate buffer for 2 hours. Samples were then dehydrated in acetone (30, 50, 70, 90, and 100%) for 15 minutes each. Fixation and dehydration on all plant tissue at the same time and was performed at 4°C. Samples were then brought to room temperature with the last dehydration step. Tissue was embedded over 24 hours by adding Spurr's resin, and then polymerized for 12 hours at 70°C. Thin sectioning was performed using a Porter Blum MT-2 ultramicrotome and were stained for 7 minutes in 1% (w/v) uranyl acetate and for 2 minutes in 0.2% (w/v) lead acetate. Thin sections were imaged using an AEI electron microscope.

Bacteria and yeast cell strains. Escherichia coli strain DH5α (F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) was cultured in Luria-Bertani (LB) broth Miller (EMD Biosciences) medium with the appropriate antibiotic and was used for cloning and propagation of plasmids. *Saccharomyces cerevisiae* strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3, MEL1GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2, URA3::MEL1*$_{UAS}$-*MEL1*$_{TATA}$-

lacZ) [15] was obtained from Clontech Laboratories, Inc. (Mountain View, CA, USA) and was cultured in yeast extract/peptone/dextrose (YPD) (1% (w/v) yeast extract (Fisher Scientific), 2% (w/v) peptone (Fisher Scientific), 2% (w/v) D-glucose (Fisher Scientific)) medium or synthetic complete drop out (SD) medium (Difco) lacking tryptophan and leucine for selection and maintenance of plasmids.

Plasmid constructs for yeast two-hybrid system. Amplification of DNA encoding the amino terminal sequences of PAA1 and PAA2 was performed by PCR using Expand High Fidelity polymerase (Roche). Oligonucleotide primer sequences used to generate DNA fragments and sequencing of plasmids for PAA1(N) are 5'-gggaattccatatgtcggagagtggtgattccaagtca -3' (forward) and 5'-cgcggatccgctcgcggccactctctttaagcg -3' (reverse) and for PAA2(N) are 5'-gggaattccatatgatcgaatctgtgaaatccattacga -3' (forward) and 5'-cgcggatcccacggttcctgctcttaacaagcaa -3' (reverse). Genomic DNA isolated from wildtype *Arabidopsis* plants was used as a template for fragment amplification. The coding region of PAA1(N) and PAA2(N) was cloned in frame into pGBKT7 and/or pGADT7 vectors (Matchmaker™ Yeast Two-Hybrid System 3, Clontech) using the *Nde*I and *Bam*HI restriction sites.

Yeast two-hybrid spot assay. Yeast cells were co-transformed with a plasmid containing an activation domain fusion and one containing a binding domain fusion using the lithium acetate (LiAc) method [16]. Selection of the transformants was performed on 1.5% agar plates containing the appropriate

dropout medium (-Leu -Trp) and several individual colonies were purified for spot assays. Liquid cultures of yeast co-transformed with cloned pGAD and pGBK vectors were propagated in media lacking leucine and tryptophan for selection of both plasmids for 24 hours at 30° C, shaking at 260 RPM. The optical denisity of yeast cultures were measured at 600nm using a DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter, Inc.) and diluted to 0.1 OD_{600}, then further diluted to 0.01 OD_{600} for spot assays. An 8-channel pipettor was used to transfer 3 µL of each dilution onto three separate agar plates containing SD minimal medium lacking tryptophan, leucine or on medium lackinig tryptophan, leucine, histidine, and adenine or on medium lacking tryptophan, leucine, histidine, and adenine supplemented with 2.5 mM 3-amino-1,2,4-triazole (Sigma). Agar plates were then incubated in 30° C for five days and each assay was repeated in at least three independent experiments.

β-galactosidase activity assays. The procedure for quantitative estimates of yeast two-hybrid interactions using β-galatcosidase was modified from the Yeast Protocol Handbook (Clontech) [16]. Liquid cultures of yeast co-transformed with cloned pGAD and pGBK vectors were propagated in the proper dropout medium for selection of both plasmids and supplemented with bathocuproine disulfonic acid (BCS) (Sigma-Aldrich) for 48 hours at 30° C, shaking at 260 RPM. Liquid β-galactosidase activity assays were then carried out following the Clontech protocol using chlorophenol red-β-D-galactopyranoside (CPRG) (BMCC) as the substrate [16]. Each culture was measured in triplicate for an individual experiment and three independent

experiments were performed. β-galactosidase Miller units [17,18] were calculated and quantities are presented as average values ± mean standard deviations for the three experiments combined. The pACT-KIPK and pAS1-KCPB(N) constructs were obtained from A.S.N. Reddy (Colorado State University, CO, USA) and the interaction between these proteins has been described [19].

In silico sequence analysis. Gene and protein information was obtained through The Arabidopsis Information Resource (TAIR) database. Sequence alignments were performed using the CLUSTAL W (1.83) multiple sequence alignment tool [20]. Plasmid DNA was sequenced in two directions using the forward and reverse gene specific primers used in cloning and/or the forward and reverse plasmid specific primers. Sequencing was performed by Macromolecular Resources (Colorado State University, Fort Collins, CO, USA).

3. Results

In order to investigate how mutations affect other proteins involved in copper homoestasis, wildtype, *paa1-3*, and *paa2-1* plants were grown for 14 days on ½ MS media and ½ MS media supplemented with various copper concentrations. Tissue was then harvested and proteins were extracted for SOD activity assays and immunoblot analysis. Manganese SOD and HSP70 were used as loading controls in all immunoblot analysis. It has been demonstrated in the past that regulation of FeSOD and CSD2 is based on the

availability of their cofactors, especially the availability of copper within the cell [12]. In this study, the addition of 0.5 - 1 μM $CuSO_4$ to the media caused a switch from the use of FeSOD to CSD2 in the chloroplast (Figure 1). FeSOD is down regulated in both of the *PAA* mutants, but is more evident in *paa1-3* compared to *paa2-1*. In most copper treatments CSD2 expression is noticeably elevated in *paa2-1* compared to wildtype plants, whereas *paa1-3* has a slightly lower expression. The expression of CSD1 did not vary between plants as greatly as CSD2. In the control treatment both mutants had a higher expression of CSD1 than wildtype. The same trend was detected in the 0.5 μM and 1 μM copper treatments; however the difference between lines diminishes at copper concentrations higher than 1 μM. Superoxide dismutase activity correlated with the protein expression levels of FeSOD, CSD1, and CSD2. The differences in SOD activity between the growth conditions can be attributed to a variance in protein abundance (Figure 1B) which may be explained by a change in transcript levels. The CCS protein followed the same expression profile as CSD1 in all plants that were tested, with an elevated expression in the *PAA* mutants at low copper concentrations. Plastocyanin accumulation in wildtype plants increased somewhat as copper concentrations increased. This direct correlation between plastocyanin expression and copper supply was also present in the *PAA* mutant plants. However, in most treatments a considerably lower amount of plastocyanin was detected in *paa1* and *paa2* compared to wildtype.

The chloroplast contains two important targets, CSD2 and PC, for copper and plastocyanin is one of the most abundant proteins in

photosynthetic tissue. With the absence of chloroplasts in root tissue we might expect a difference in copper homeostasis regulation between tissue types. In order to investigate this, wildtype, *paa1-3*, and *paa2-1* plants were grown for 14 days in ½ MS containing various copper concentrations. When tissue was harvested roots and shoots were dissected separately and total soluble protein was extracted for immunoblot analysis. FeSOD expression was detected at a higher level in the shoots compared to the roots in all plants and was only present under very low copper supply (Figure 2). Although expression of FeSOD was seen in all plants wildtype exhibited a much higher amount of the protein (especially in shoot tissue) compared to *paa1-3* and *paa2-1*. In fact, there was almost an absence of FeSOD in the *paa1-3* mutant in the control treatment. Expression levels of CSD1 and CSD2 was detected in different levels in the varying tissue types. CSD1 expression levels were higher in root tissue compared to shoot tissue, while CSD2 expression levels were higher in shoot tissue compared to roots. Interestingly, CSD1 protein quantity in the roots was similar in all treatments and all plant types; however CSD1 amounts in shoot tissue varied slightly between wildtype and the *PAA* mutants in some treatments and slightly increased as copper supply increased. Increased levels of CSD2 were detected *paa2-1* compared to wildtype and *paa1-3*. This elevated level of CSD2 protein in *paa2-1* was most apparent in the control treatment were there is very little copper present in the media. Though as copper supply increased, CSD2 protein increased in wildtype plants and levels became similar *paa2-1*. However, CSD2 remained low in *paa1-3* plants across copper concentrations. Similar to SOD expression, CCS protein levels were

detected in both roots and shoots, although root tissue exhibited lower amounts of the protein compared to shoot tissue. Expression of CCS was similar in wildtype and mutant plants and appeared consistent across copper treatments in root tissue. However, in shoot tissue CCS seemed to increase slightly as copper supply increased. This was most noticeable between ½ MS and 1 μM copper treatments. As expected, plastocyanin protein was only detected in photosynthetic tissue and at a lower amount in the *PAA* mutants compared to wildtype.

Growth rate and chlorophyll fluorescence phenotypes have been previously described for the *paa1-1*, *paa1-3*, and *paa2-1* mutants [7,8]. Furthermore, in this study we have established that CCS, CSD1, CSD2, as well as plastocyanin protein expression are affected by mutations in the *PAA* genes. With such severe visual as well as biochemical phenotypes it seems likely that a mutation in *PAA1* and *PAA2* may also affect the ultrastructure of the chloroplasts. To investigate this, wildtype, *paa1-1*, *paa1-3*, and *paa2-1* plants were grown on ½ MS media and ½ MS media supplemented with 10 μM $CuSO_4$ for 14 days. Leaf tissue samples were taken for analysis of chloroplast ultrastructure by transmission electron microscopy. Interestingly, wildtype plants grown on ½ MS media display an unequal distribution of thylakoid membranes (Figure 3). In all chloroplasts, thylakoid membranes were unevenly distributed and were found arranged to one side of the plastid cross-sections. In the copper treatment, wildtype plastids showed a more even distribution of thylakoid membranes. This difference in arrangement of membranes was not observed to the same extent in any of the mutants in this

investigation. Wildtype plants also displayed distinct stacking of the thylakoid membranes that form grana. We observed a difference of chloroplast structure in the *paa1* and *paa2* mutants when compared to wildtype plants we are classifying as a structural change due to stress (Figures 4-6). This damage looks similarly to starch granules that would accumulate between the thylakoid membranes within the plastid. However, little accumulation of starch granules was observed in all plant lines in this investigation and the *PAA* mutants are thought to be deficient in starch accumulation since photosynthetic activity is so severely affected (M. Pilon, unpublished data). Additionally, the *paa1* mutants (specifically *paa1-3*) displayed decreased stacking or disassociation of the thylakoid membranes in the copper treatment when compared to the other plant lines (Figure 4 and 5).

The amino termini of PAA1 and PAA2 contain an ATX-like structural domain (Figure 7) and it is possible that these domains act similarly to copper chaperones in accepting and delivering copper. The yeast two-hybrid technique was utilized to investigate if PAA1 and PAA2 could interact with each other via their ATX-like domains. In order to detect PAA1 and PAA2 expression within a variety of systems, including yeast, antisera were produced from purified recombinant proteins (amino termini only without affinity tags). Encoded regions of PAA1 and PAA2 used for antisera production as well as yeast two-hybrid analysis can be found in Figure 7. Figure 8 displays the purified proteins (w/ tags) and illustrates that antisera are capable of detecting as little as 1 ng of purified protein. Additionally, no cross reaction of antibodies with PAA1 and PAA2 was detected (data not shown). Yeast two-

hybrid results indicated that there is an interaction between PAA1(N) and PAA2(N), however those same domains do not interact with themselves (Figure 9).

Beta-galactosidase activity was measured to obtain a quantitative estimate of the yeast two-hybrid interactions. In this assay, the PAA1(N)/PAA2(N) interaction was 10-fold greater than the empty vector control, whereas the PAA2(N)/PAA1(N) interaction was only approximately 2-fold greater than the same control (Figure 10A). This difference in interaction between these reciprocal combinations is most likely do to the effect of the yeast two-hybrid tag on the protein charge and/or confirmation. To test if this interaction is copper dependent, a copper chelator (BCS) was added to the growth media and β-galactosidase activity was measured again. The KCBP(N)/KIPK interaction that is not involved in copper homeostasis was used as a control (Figure 10B). The β-galactosidase activity of the PAA1(N)/PAA2(N) interaction decreased approximately 50% at 500 μM BCS and decreased 70% at 1000 μM BCS concentration (Figure 10C). There was also a decrease in β-galactosidase activity when the reciprocal interaction, PAA2(N)/PAA1(N), was tested although it was not as dramatic. At 500 μM BCS activity decreased about 20%, whereas a decrease of 30% was seen at 1000 μM BCS (Figure 10D).

4. Discussion

Mutations in the *PAA1* and *PAA2* genes dramatically affect *Arabidopsis* copper homeostasis. However, roots and shoots are affected differently within

the *paa1-3* and *paa2-1* plants. FeSOD and CSD2 are both localized to the chloroplast and are seen at higher expression levels in shoot tissue versus root tissue. CSD1 is a cytosolic protein and therefore is detected in both root and shoot tissue, however at slightly varying amounts. Protein levels of CSD1 in roots were consistent between wildtype and mutant plants across all treatments. Since green chloroplasts are not formed in roots, cellular copper supply is diverted to other copper targets. Additionally, there is evidence of a slightly higher copper concentration in root cells compared to shoot cells [5]. With this additional buildup of copper ions in the cytosol CSD1 expression is consistently high to sequester excess ions within the cell. Alternatively, in shoot tissue the cell has to regulate copper transport into the chloroplast for plastocyanin and as a result at low copper concentration there are fewer copper ions in the cytosol. This would explain a small decrease in CSD1 expression in ½ MS media. CSD2 expression in *paa2-1* whole plant tissue and shoot tissue showed a dramatic increase, which could be attributed to the defect in copper transport at the thylakoid membrane. As copper ions are transported into the chloroplast and accumulated in the stroma, ion concentrations decrease in the cytosol and subsequently lead to a decrease in CSD1 expression in the same mutant. The expression of CCS was slightly elevated in whole plant tissue of *paa1-3* and *paa2-1* plants at low copper concentrations which correspond to SOD expression. It is currently unknown what the regulatory elements are for gene transcription of *CCS*, *CSD1*, and *CSD2*, however expression of CCS and CSD1 are similar and may have the same regulatory element controlling transcription of both genes.

It has been determined that mutations in the *PAA1* and *PAA2* genes create defects in photosynthetic activity that dramatically affects both growth rate and chlorophyll fluorescence [7,8]. In addition, this investigation has demonstrated that these same mutations can contribute to stress-related structural change to the structure of the chloroplast. This structural change may be caused by either the buildup of superoxides from photosynthetic activity or in some cases a buildup of toxic copper ions in the stroma, especially in *paa2-1*. Media, such as MS, has very low levels of copper (approximately 0.05 μM) which may be deficient to plants. The presence of FeSOD protein in ½ MS also suggests that plants may not be acquiring a sufficient amount of the ion for proper growth. The uneven distribution of thylakoid membranes in wildtype plants in ½ MS copper may be a result of the ion deficiency, especially if PAA1 and PAA2 transporters directly interact with one another for copper delivery to plastocyanin.

Yeast two hybrid is a technique that is often used to establish possible interactions between proteins involved in copper transport [5,21-23], however interactions have only been discovered between transporters and copper chaperones. In this study, we show that the amino terminal domains of PAA1 and PAA2 can interact with each other *in vitro*. Both domains possess a copper binding motif MxCxxC as well as the βαββαβ structure similar to the ATX copper chaperone. It has been proposed that the interactions occur between the ATX-like domain of a transporter and the ATX-like domain of a copper chaperone; however this is the first instance in which an interaction between two transporters has been demonstrated. In general, charge, size,

and confirmation of proteins can contribute to protein-protein interactions. In addition to the conformation of ATX-like folds of the PAA1 and PAA2 amino termini, we believe that charge may also contribute to the interaction between the transporters. The interaction between these transporters also seems to be copper dependent, similar to other interactions shown with copper homeostasis proteins [21,24,25].

Topologies of the PAA1 and PAA2 transporters have not yet been resolved, however P-type ATPases require the presence of ATP to change conformation and translocate ions. Therefore, it is predicted that the N- and C-terminal domains along with a large cytosolic loop containing the ATP binding domain of PAA2 are located in the stroma. Nevertheless, ATP is present within the stroma as well as the cytosol and the orientation of PAA1 could be either direction. Some scientists have speculated that the P-type ATPases are faced in a direction in which the domain containing the copper binding motif accepts the copper ion for transport across a membrane. This would result in the cytosolic location of the PAA1 amino terminus. However, some investigations in the cyanobacteria, *Synechocystis*, have revealed that the CtaA transporter amino terminus in located within the cytosol [26]. This provides important evidence that the heavy metal binding domain accepting copper can be orientated the opposite direction that was originally predicted.

Based on the data acquired in this investigation we propose a new model for copper transport within the chloroplast for delivery to plastocyanin (Figure 11). In this model, we suggest that there may be a direct interaction between the amino terminal domains of PAA1 and PAA2 without the

requirement of a copper chaperone intermediary. Currently, only one copper chaperone within the chloroplast has been identified and characterized, CCS, and has been shown to deliver copper to CSD2 [12,27]. Several other putative copper chaperones have been identified as candidates for chloroplastic transport from PAA1 to PAA2; however investigations have not yet led to experimental data to place them in this transport pathway. Further investigations must be made to resolve the topologies of these transporters, however *in vivo* data will be difficult to obtain. Membrane transporters are difficult to work with *in planta* especially when working with fusion tags. Without proper insertion of the protein in its given membrane topology is unable to be determined. Additionally, we noted differences in thylakoid organization in wildtype plants between treatments which could be the consequence of the interaction between PAA1 and PAA2 to facilitate transport to plastocyanin. Alternatively, we do not believe that this membrane organization is caused by the concentration of copper within the chloroplast. If this were the case, *paa1* mutants would have chloroplasts with an uneven distribution of thylakoids due to the deficiency of copper ions and *paa2* plants would display thylakoids that are separated from the envelope due to the accumulation of the metal ion within the stroma.

In this investigation, we have further characterized the *paa1-1*, *paa1-3*, and *paa2-1* mutant plants and determined that mutations affect copper homeostasis differently in root and shoot tissue. We have also identified a change in chloroplast ultrastructure in *paa1* and *paa2* mutant plants that is most likely caused by stress-related damage. Additionally, we describe a novel

interaction between two separate P-type ATPase transporter domains which leads us to a newly proposed model for chloroplast copper transport.

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References

[1] Schubert, M., Petersson, U.A., Haas, B.J., Funk, C., Schroder, W.P. and Kieselbach, T. (2002). Proteome map of the chloroplast lumen of Arabidopsis thaliana. J Biol Chem 277, 8354-65.

[2] Zhang, L., McSpadden, B., Pakrasi, H.B. and Whitmarsh, J. (1992). Copper-mediated regulation of cytochrome c553 and plastocyanin in the cyanobacterium Synechocystis 6803. J Biol Chem 267, 19054-9.

[3] Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D. and Van Montagu, M. (1995). Molecular characterization of a putative Arabidopsis thaliana copper transporter and its yeast homologue. J Biol Chem 270, 28479-86.

[4] Kliebenstein, D.J., Monde, R.A. and Last, R.L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118, 637-50.

[5] Andres-Colas, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker, J.R., Puig, S. and Penarrubia, L. (2006). The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J 45, 225-36.

[6] Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker J.R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97, 383-93.

[7] Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K.K. and Pilon, M. (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15, 1333-46.

[8] Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M. and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17, 1233-51.

[9] Marschner, H. (1995) Mineral nutrition of higher plants, Academic Press. London ; San Diego.

[10] Murphy, A. and Taiz, L. (1995). Comparison of metallothionein gene expression and nonprotein thiols in ten Arabidopsis ecotypes. Correlation with copper tolerance. Plant Physiol 109, 945-54.

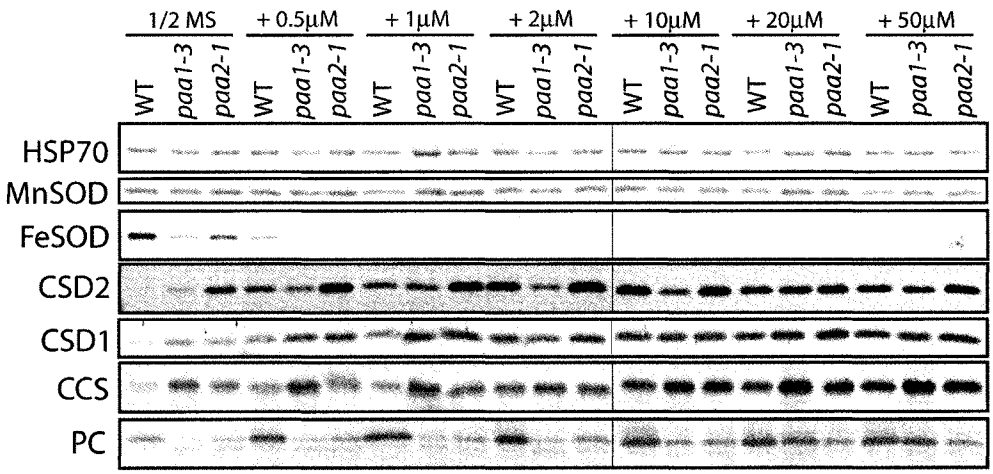
[11] Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

[12] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., Andres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Lett 579, 2307-12.

[13] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-54.

[14] Beauchamp, C. and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44, 276-87.
[15] James, P., Halladay, J. and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425-36.
[16] Clontech. (2001) Yeast Protocols Handbook. Mountain View, CA.
[17] Miller, J.H. (1972) Experiments in Molecular Geneticsed.^eds). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[18] Miller, J.H. (1992). In A Short Course in Bacterial Genetics ed.^eds), pp. 74. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[19] Day, I.S., Miller, C., Golovkin, M. and Reddy, A.S. (2000). Interaction of a kinesin-like calmodulin-binding protein with a protein kinase. J Biol Chem 275, 13737-45.
[20] Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G. and Thompson, J.D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31, 3497-500.
[21] van Dongen, E.M., Klomp, L.W. and Merkx, M. (2004). Copper-dependent protein-protein interactions studied by yeast two-hybrid analysis. Biochem Biophys Res Commun 323, 789-95.
[22] Puig, S., Mira, H., Dorcey, E., Sancenon, V., Andres-Colas, N., Garcia-Molina, A., Burkhead, J.L., Gogolin, K.A., Abdel-Ghany, S.E., Thiele, D.J., Ecker, J.R., Pilon, M., Penarrubia, L. (2007). Higher plants possess two different types of ATX1-like copper chaperones. Biochemical and Biophysical Research Communications 354, 385-390.
[23] Lim, C.M., Cater, M.A., Mercer, J.F. and La Fontaine, S. (2006). Copper-dependent interaction of dynactin subunit p62 with the N terminus of ATP7B but not ATP7A. J Biol Chem 281, 14006-14.
[24] Huffman, D.L. and O'Halloran, T.V. (2000). Energetics of copper trafficking between the Atx1 metallochaperone and the intracellular copper transporter, Ccc2. J Biol Chem 275, 18611-4.
[25] Harvie, D.R., Andreini, C., Cavallaro, G., Meng, W., Connolly, B.A., Yoshida, K., Fujita, Y., Harwood, C.R., Radford, D.S., Tottey, S., Cavet, J.S., Robinson, N.J. (2006). Predicting metals sensed by ArsR-SmtB repressors: allosteric interference by a non-effector metal. Mol Microbiol 59, 1341-56.
[26] Cavet, J.S., Borrelly, G.P. and Robinson, N.J. (2003). Zn, Cu and Co in cyanobacteria: selective control of metal availability. FEMS Microbiol Rev 27, 165-81.
[27] Chu, C.C., Lee, W.C., Guo, W.Y., Pan, S.M., Chen, L.J., Li, H.M. and Jinn, T.L. (2005). A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. Plant Physiol 139, 425-36.

A.



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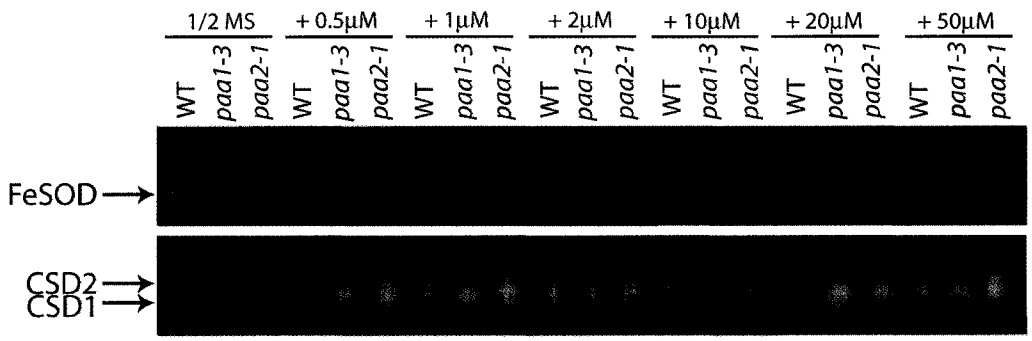
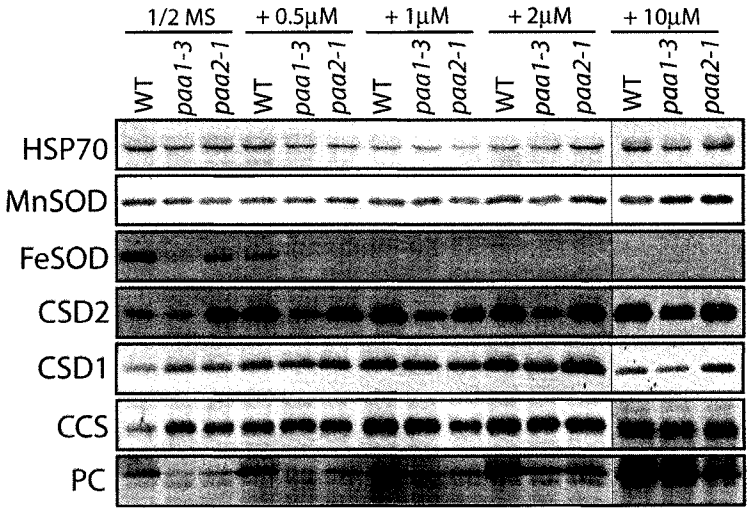


Figure 1. Effect of *PAA* mutations on copper homeostasis protein expression and activity. Immunoblot analysis of wildtype (WT), *paa1-3*, and *paa2-1* plants grown for 14 days in ½ MS and ½ MS supplemented with 0.5 - 50 μM $CuSO_4$ (Panel A). A total of 10 μg of soluble protein from whole plant tissue was resolved on 12.5% SDS-PAGE gels for protein expression. SOD activity was analyzed using the same tissue (Panel B). A total of 10 μg of soluble protein was resolved on a 15% NATIVE-PAGE gel for FeSOD analysis and on a 10-20% Tris-HCL gradient gel for analysis of CSD1 and CSD2 activity. HSP70 and MnSOD proteins were used as loading controls.

A. Shoots



B. Roots

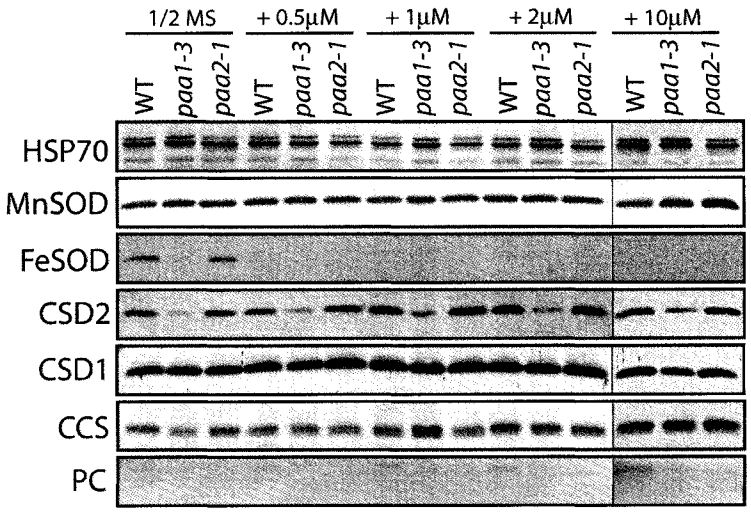


Figure 2. *PAA* mutations affect copper homeostasis protein levels differently in roots and shoots. Immunoblot analysis of wildtype (WT), *paa1-3*, and *paa2-1* plants grown for 14 days in ½ MS and ½ MS supplemented with 0.5 - 10 μM $CuSO_4$. Tissue was dissected and harvested separately for analysis and a total of 10 μg of soluble protein from root or shoot tissue was resolved on 12.5% SDS-PAGE gels for protein expression. HSP70 and MnSOD proteins were used as loading controls.

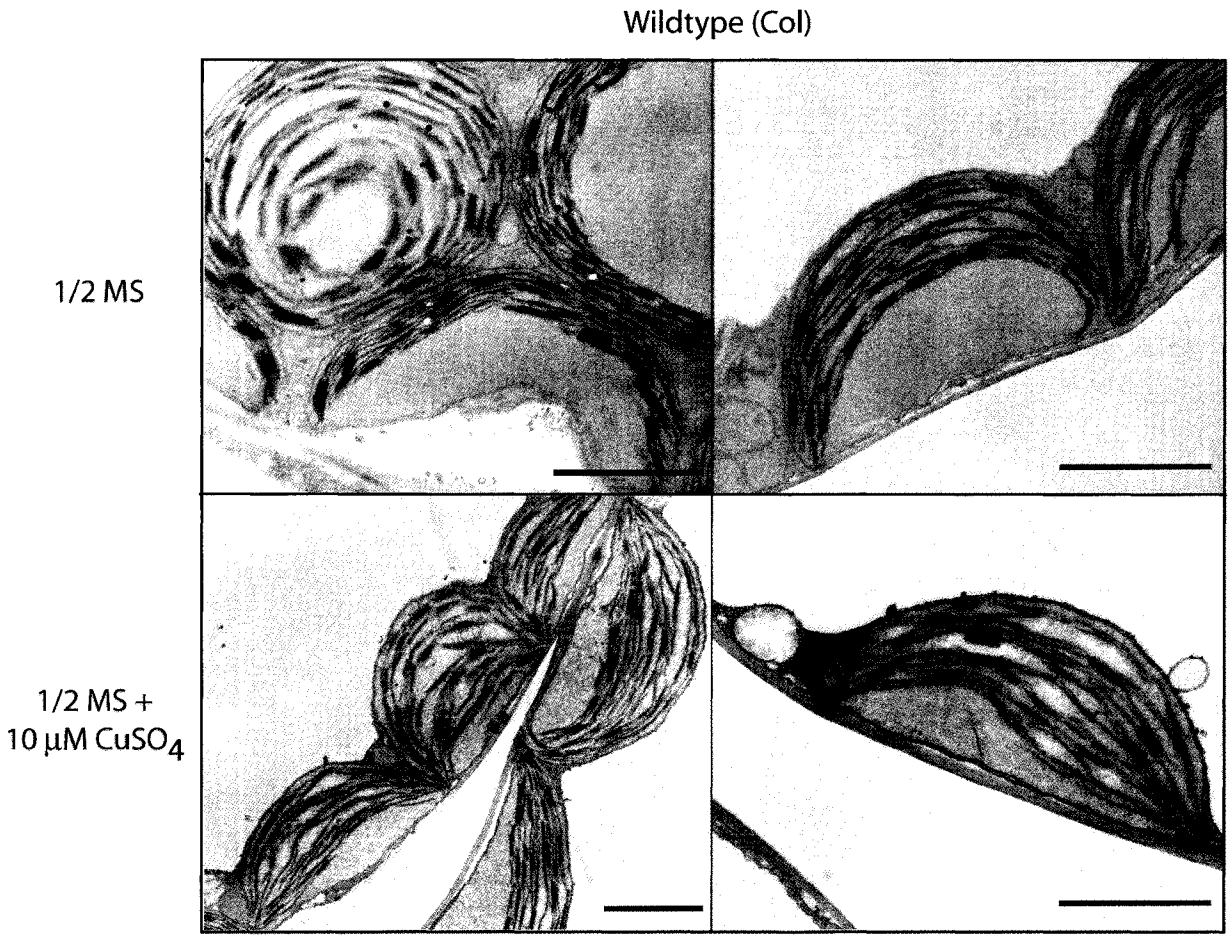


Figure 3. Chloroplast structure of wildtype plants in varying copper concentrations. Wildtype, *paa1-1*, *paa1-3*, and *paa2-1* plants were grown for 14 days in ½ MS and ½ MS supplemented with 10 µM $CuSO_4$. Vegetative tissue was harvested and prepared for transmission electron microscopy analysis (Scale bars = 2 µm).

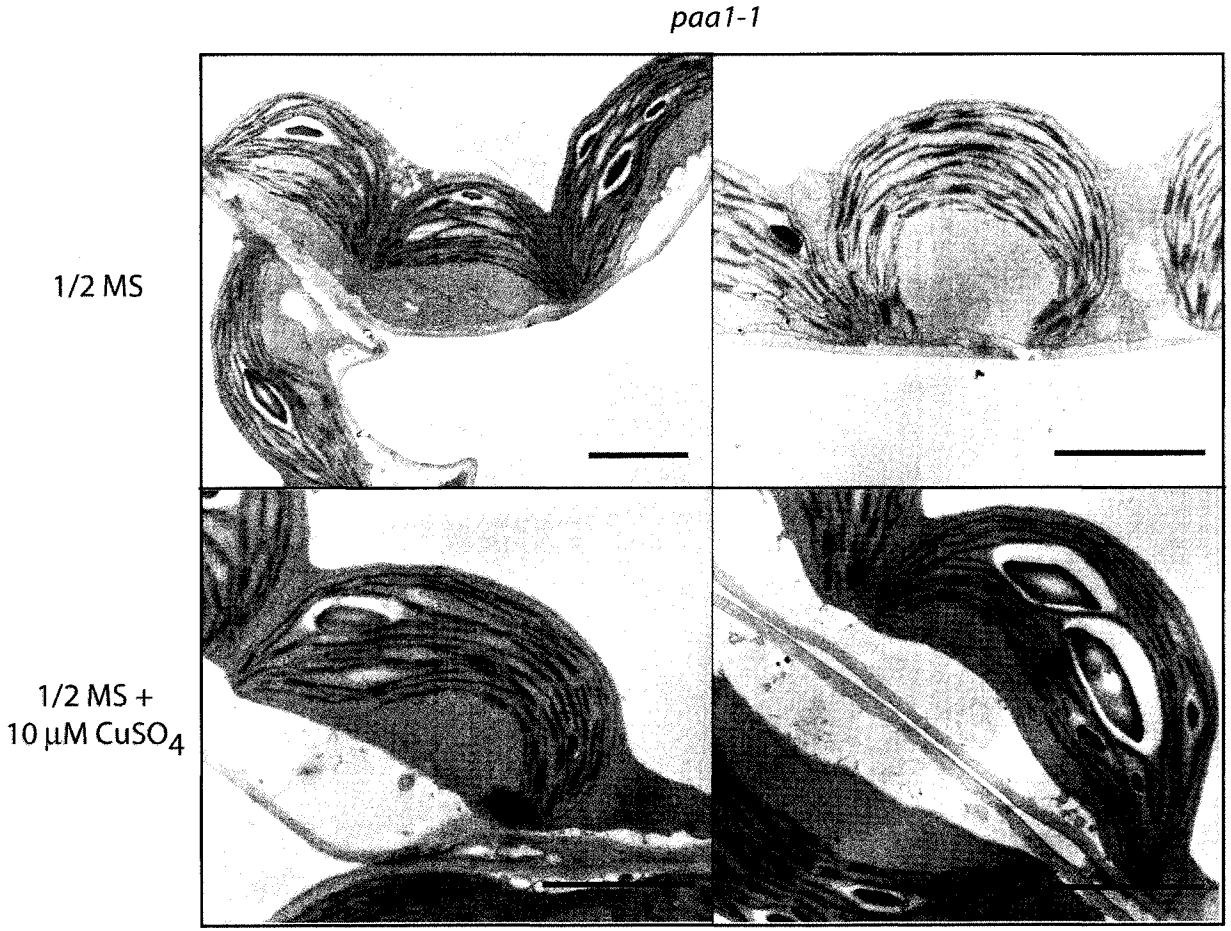


Figure 4. Chloroplast structure of *paa1-1* plants in varying copper concentrations. Wildtype, *paa1-1*, *paa1-3*, and *paa2-1* plants were grown for 14 days in ½ MS and ½ MS supplemented with 10 μM $CuSO_4$. Vegetative tissue was harvested and prepared for transmission electron microscopy analysis (Scale bars = 2 μm).

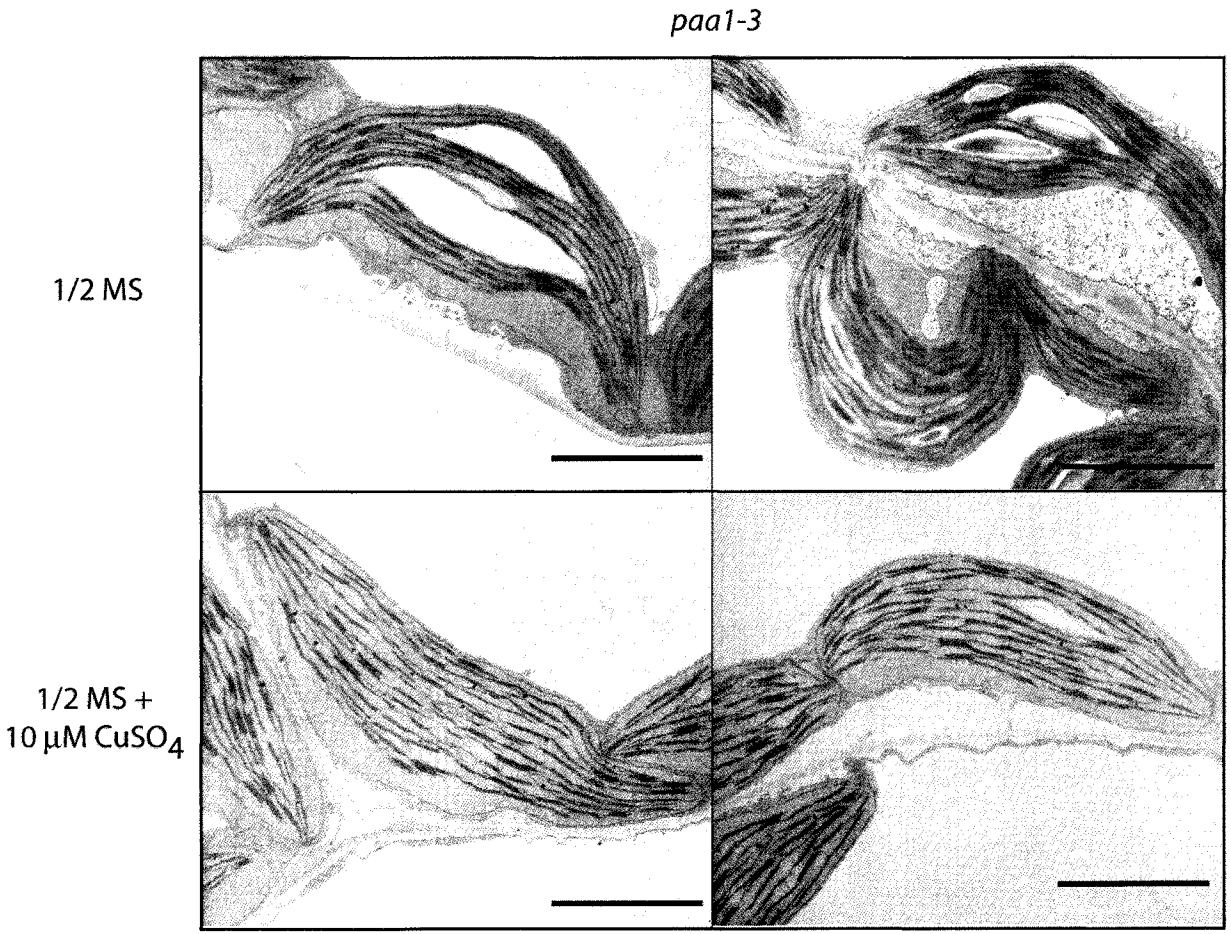


Figure 5. Chloroplast structure of *paa1-3* plants in varying copper concentrations. Wildtype, *paa1-1*, *paa1-3*, and *paa2-1* plants were grown for 14 days in ½ MS and ½ MS supplemented with 10 µM $CuSO_4$. Vegetative tissue was harvested and prepared for transmission electron microscopy analysis (Scale bars = 2 µm).

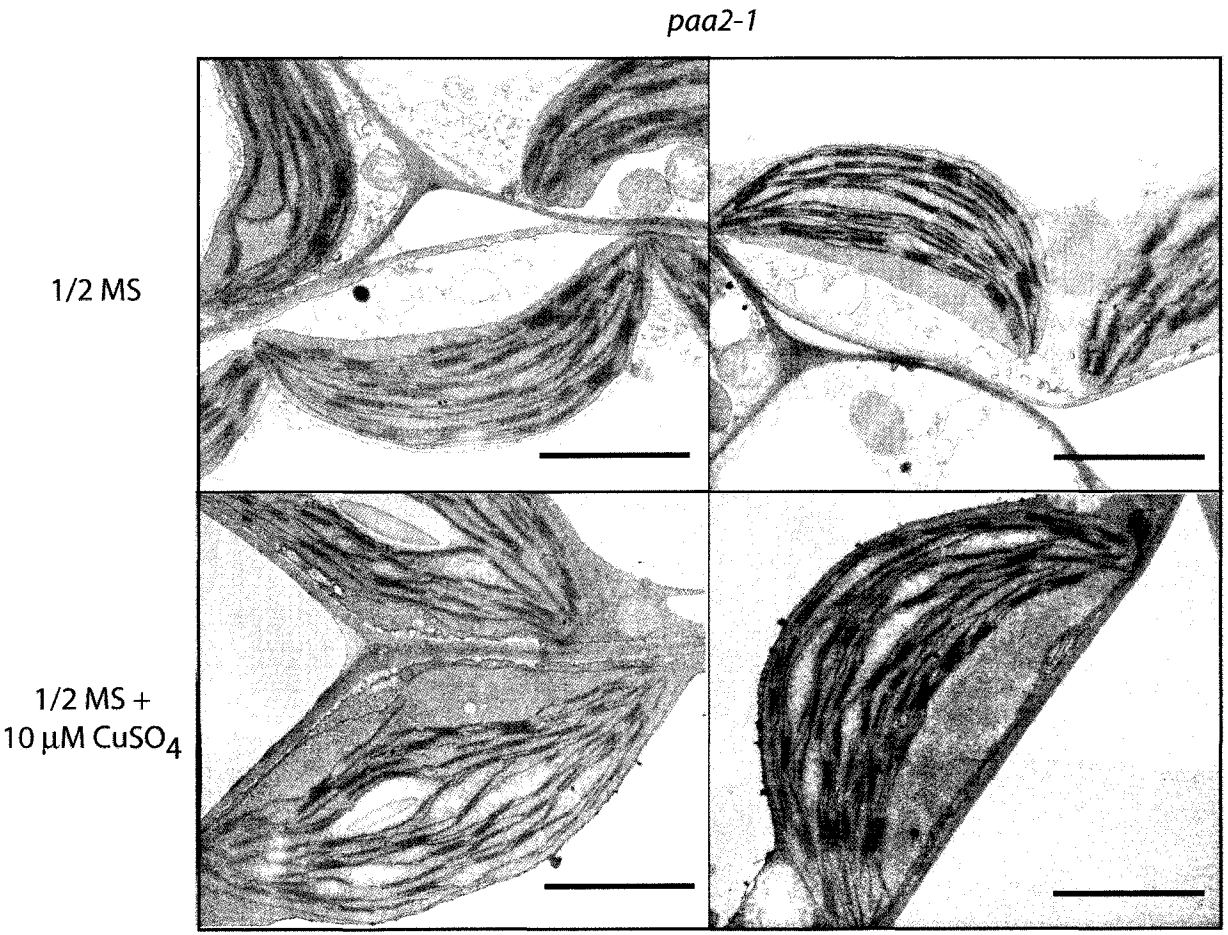


Figure 6. Chloroplast structure of *paa1-3* plants in varying copper concentrations. Wildtype, *paa1-1*, *paa1-3*, and *paa2-1* plants were grown for 14 days in ½ MS and ½ MS supplemented with 10 µM $CuSO_4$. Vegetative tissue was harvested and prepared for transmission electron microscopy analysis (Scale bars = 2 µm).

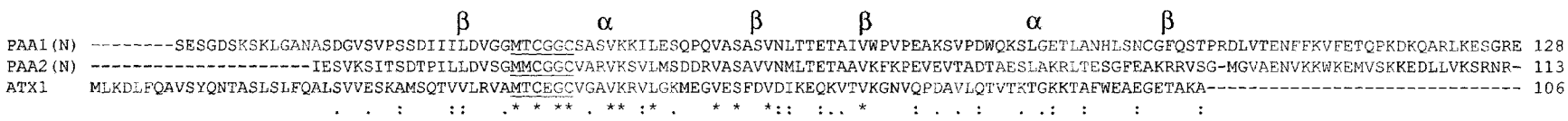


Figure 7. PAA1(N) and PAA2(N) contain ATX-like domains. Sequence and structural similarity of PAA1(N), PAA2(N), and the ATX copper chaperone. Colors represent alpha helixes (red) and beta (blue) sheets, underlined sequence depict heavy metal binding domain, asterisks represent identical residues, and dots symbolize residue similarity.

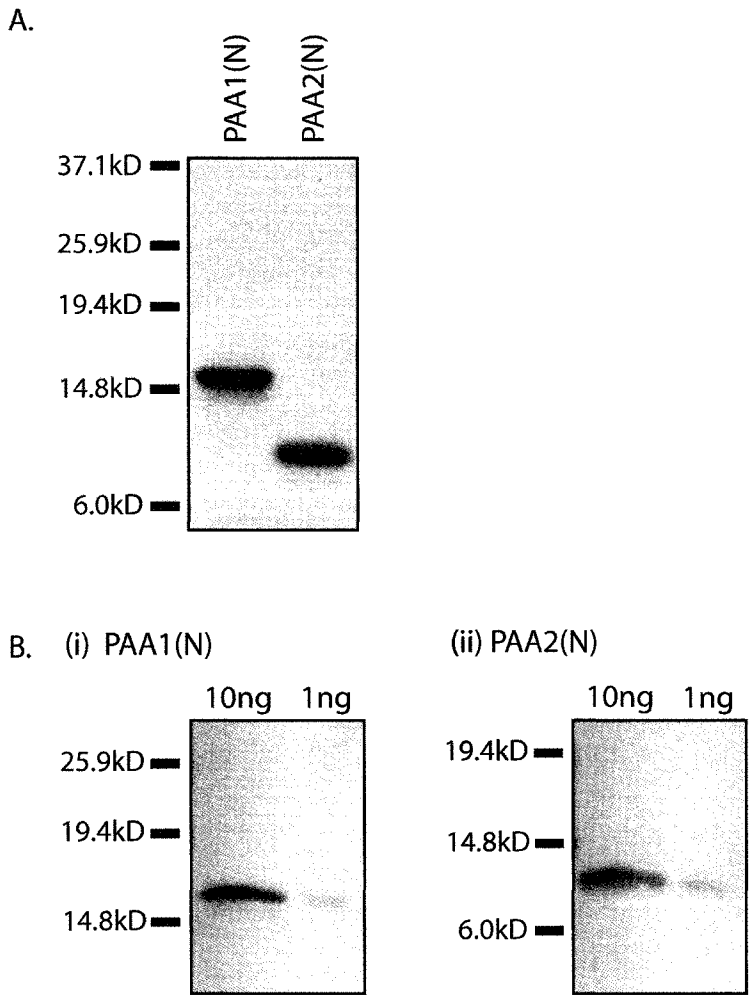


Figure 8. Purification of PAA1and PAA2 amino terminal domains and antisera production. Recombinant protein was expressed in *E.coli* and purified using affinity chromatography. Purified protein (5 µg) was resolved on a 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue (Panel A). Polyclonal antisera were then produced for purified amino terminal domains and can detect as little as 1 ng of purified protein (Panel B).

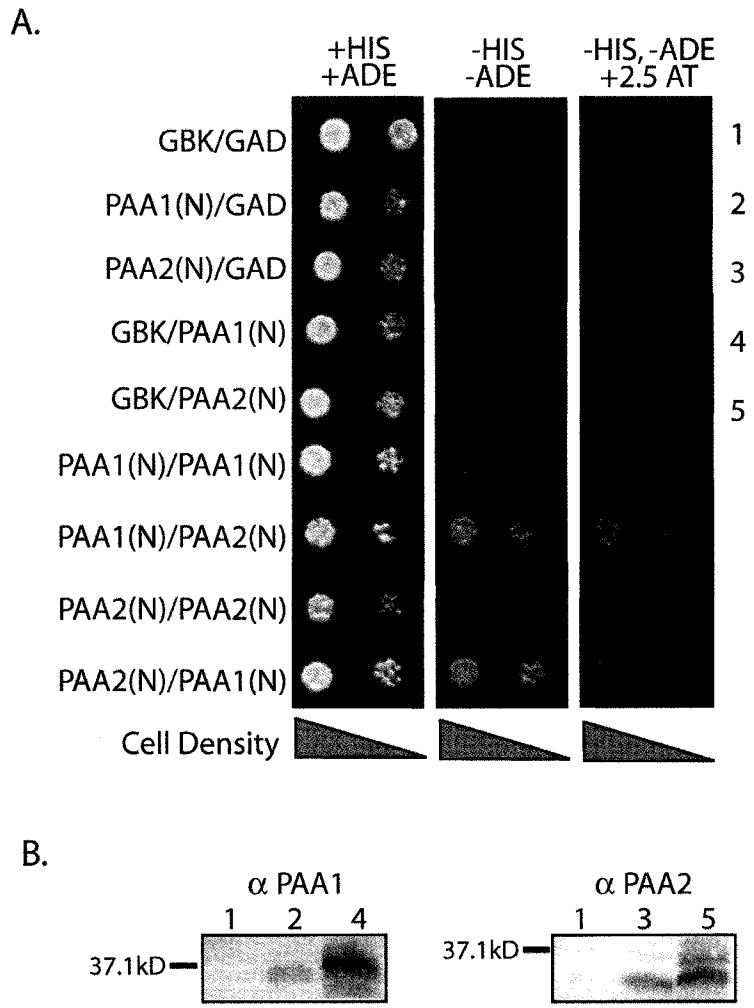


Figure 9. Interaction between PAA1(N) and PAA2(N) transporters using yeast two-hybrid. A yeast two-hybrid spot assay was conducted using the ATX-like domains of PAA1 and PAA2. AH109 yeast cells were co-transformed with pGAD- and pGBK- based plasmids containing encoding regions of both proteins. Cells were grown in liquid cultures and spotted on plates containing +HIS+ADE, -HIS-ADE, and -HIS-ADE supplemented with 2.5 mM 3-amino-1,2,4-triazole (AT) (Panel A). Fusion proteins are expressed in yeast cells (Panel B). Yeast transformants were analyzed by immunoblot analysis with PAA1(N) and PAA2(N) antibodies. Sizes of proteins correspond to the appropriate molecular weight of purified amino terminal domains fused to yeast two-hybrid tags.

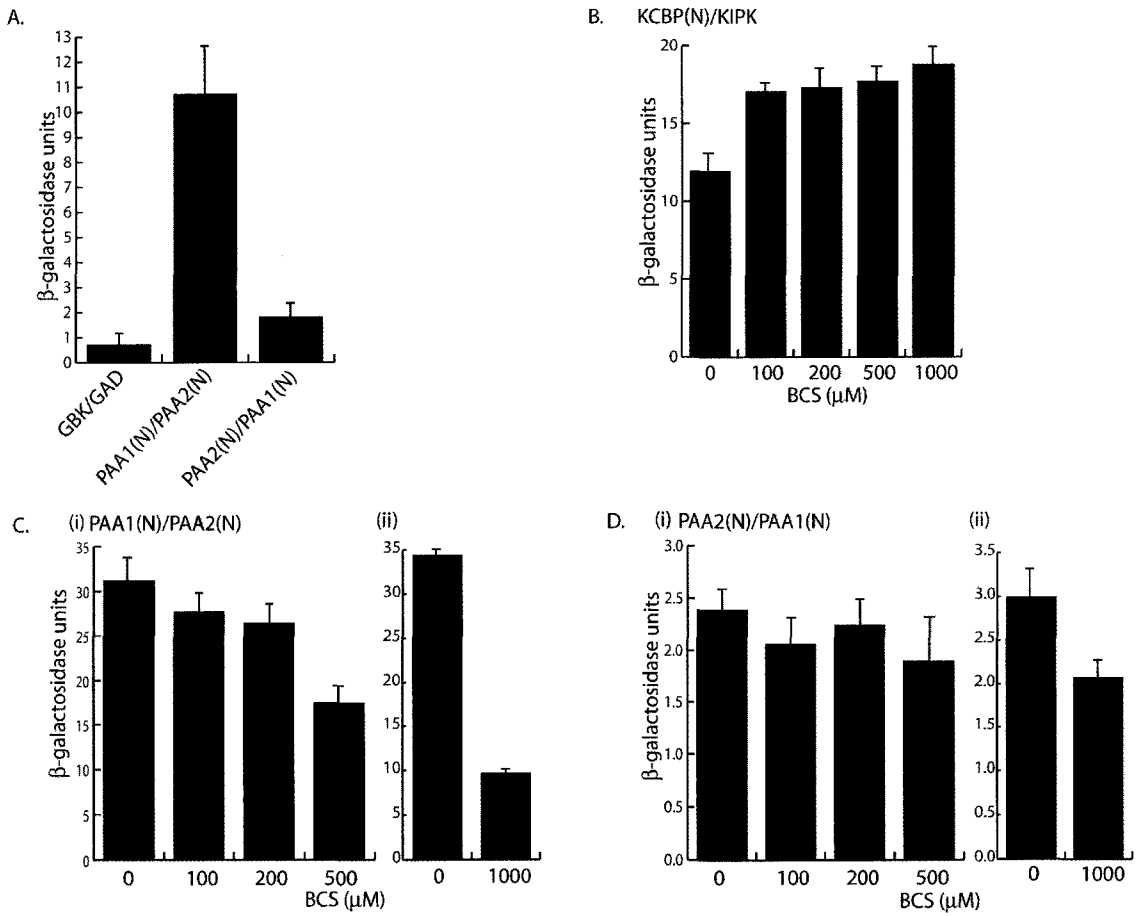


Figure 10. Quantitative estimates of the interaction between PAA1(N) and PAA2(N) and the effect of a copper chelator on the interaction. AH109 yeast cells were co-transformed and grown in liquid cultures for β-galactosidase activity measurements (Panel A). Bathocuproine disulfonic acid (BCS) was added to the media to test whether the interaction is copper dependent (Panels B-D). The KCBP(N)/KIPK interaction was used as a copper independent control.

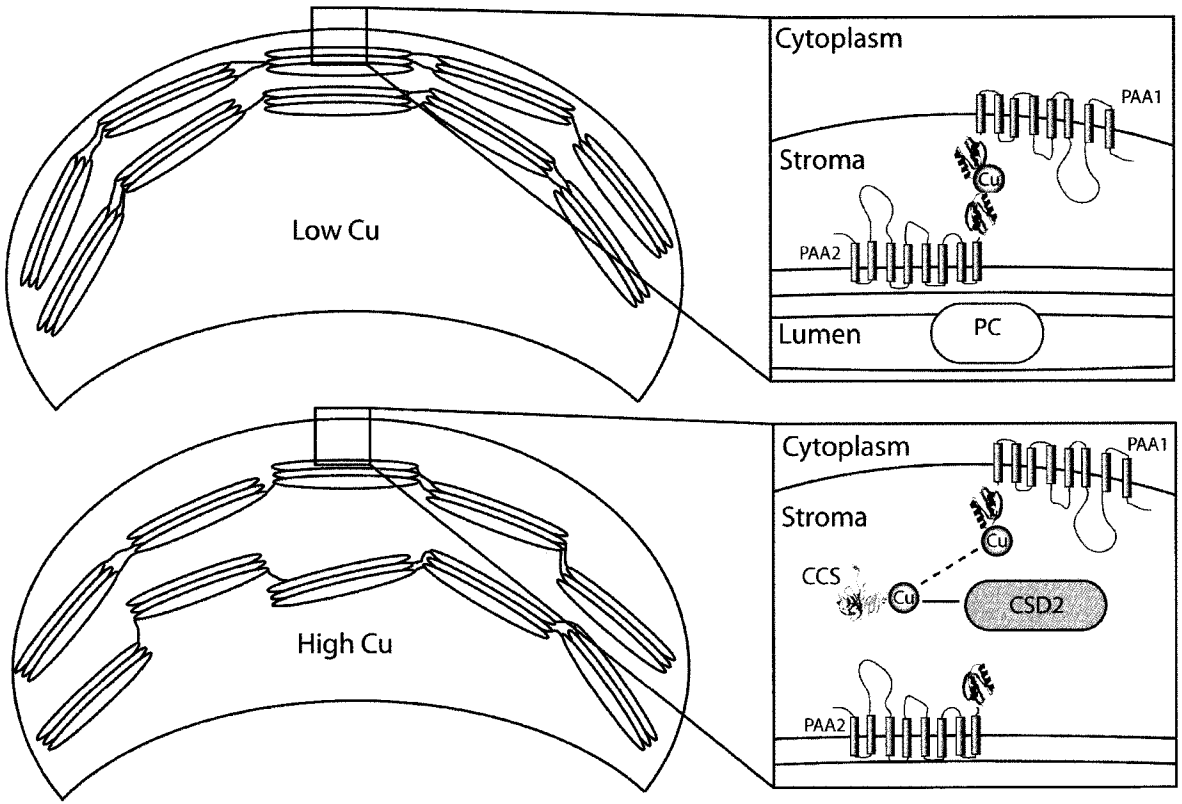


Figure 11. A new model for copper transport in the chloroplast indicating a direct interaction between the PAA1 and PAA2 transporters.

CHAPTER 3

AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7

The following chapter is found as the publication:
AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Letters, Volume 579, Issue 11, Pages 2307-2312 S. Abdel-Ghany, J. Burkhead, K. Gogolin, N. Andrés-Colás, J. Bodecker, S. Puig, L. Peñarrubia, M. Pilon

Abstract

In plant chloroplasts two superoxide dismutase (SOD) activities occur, FeSOD and Cu/ZnSOD, with reciprocal regulation in response to copper availability. This system presents a unique model to study the regulation of metal-cofactor delivery to an organelle. The *Arabidopsis thaliana* gene *AtCCS* encodes a functional homologue to yeast Ccs1p/Lys7p, a copper chaperone for SOD. The AtCCS protein was localized to chloroplasts where it may supply copper to the stromal Cu/ZnSOD. AtCCS mRNA expression levels are up-regulated in response to Cu-feeding and senescence. We propose that AtCCS expression is regulated to allow the most optimal use of Cu for photosynthesis.

Abbreviations used:

CCS, copper chaperone for Cu/Zn superoxide dismutase; Cu/ZnSOD, Cu/Zn superoxide dismutase; FeSOD, Fe superoxide dismutase; SOD, superoxide dismutase; GFP, Green Fluorescent Protein; CaMV35S, Cauliflower Mosaic Virus 35S promoter.

1. Introduction

The two major targets for copper (Cu) delivery within chloroplasts are plastocyanin in the thylakoid lumen and Cu/Zn superoxide dismutase (Cu/ZnSOD) in the stroma. Superoxide ions (O_2^-) are formed by the photoreduction of O_2 at photosystem-I [1]. Superoxide dismutase (SOD) detoxifies the potentially damaging superoxide to hydrogen peroxide (H_2O_2) and O_2; for a review see [2]. H_2O_2 is further reduced to water by other enzymes in the water-water cycle [1]. Four major SOD activities are detected in *Arabidopsis* [3]. The mitochondrial manganese SOD (MnSOD) is constitutively expressed [3]. One isoform of Cu/ZnSOD is found in the cytosol (CSD1), whereas another major isoform of Cu/ZnSOD is localized in the chloroplast stroma (CSD2) [3]. Next to Cu/ZnSOD, the stroma can also contain the activity of an FeSOD [3], which is structurally unrelated to Cu/ZnSOD [2].

To regulate micronutrient distribution but at the same time avoid metal ion-induced damage, all living organisms have evolved mechanisms to acquire and deliver cofactors to target proteins but at the same time avoid the accumulation of potentially damaging free metal ions in cells [4]. The *Arabidopsis COPT1* gene and its four homologues encode copper transporters

that may allow the entrance of Cu into cells [5-7]. Copper is imported into the chloroplast stroma by the P-type ATPase PAA1 and mutations in *PAA1* affect both stromal Cu/ZnSOD activity and PC [8]. The *Arabidopsis* genome encodes a protein with high sequence similarity to PAA1, called PAA2, which functions to move Cu into the thylakoid lumen for delivery to plastocyanin [9].

To avoid improper interactions but also to ensure that the correct delivery pathway is used, copper ions may be directed to specific targets while bound to cysteine-containing proteins known as copper chaperones; for a review see [10]. In yeast, Ccs1p/Lys7p delivers copper to the Cu/ZnSOD (Sod1p) by a direct protein-protein interaction [11,12], a function which is required to maintain the activity of reactive oxygen species sensitive enzymes involved in lysine and methionine biosynthesis [13]. Since chloroplasts are a site of oxygen production it is of interest to note that O_2 and the copper chaperone for Cu/Zn superoxide dismutase (CCS) regulate posttranslational activation of Cu/ZnSOD enzymes [14] by mediating correct disulfide formation [15]. A single gene, *AtCCS* encodes for CCS in *Arabidopsis* [16]. *Arabidopsis CCS* encodes a protein with a predicted chloroplast targeting sequence but dual localization in both cytosol and plastids was predicted if transcripts of alternative length are produced [16]. Sequences encoding CCS have also been found in other higher plants such as tomato [17], potato [18] and maize [19].

The expression and regulation of SOD isoforms in plants has been a topic of a number of studies, which showed that oxidative stress, light, and electron transport activity affect plastidic SOD isoform expression [3,8,20]. It

was recently shown that copper feeding dramatically affects the activity of FeSOD and Cu/ZnSOD isoforms, whereas the activity of MnSOD is not affected by Cu availability [9]. *Arabidopsis* plants grown on media in which Cu is not limiting express Cu/Zn SOD in the cytosol and stroma. In contrast, plants grown on low Cu media do not express Cu/ZnSOD in green tissue, which may allow more efficient Cu delivery to plastocyanin under these conditions. To allow the dismutation of superoxide an FeSOD is expressed in the stroma under Cu limitation. Interestingly, in the *paa2* mutant, defective in Cu transport to the thylakoids, an increased expression and activity of Cu/ZnSOD in the stroma was observed [9]. These observations prompted us to investigate Cu delivery to stromal Cu/ZnSOD and its regulation.

2. Materials and Methods

Plant material. Arabidopsis plants, the wildtype ecotype Colombia-0 and the mutant lines *paa1-3* [12] and *paa2-1* [13] were grown under a 16h light/8h dark cycle on soil at 23°C. For growth on agar media, seeds were surface-sterilized and sown on solid 1/2 strength Murashige and Skoog (1/2 MS) medium including 3% sucrose and 0.4% agargel [21]. The medium was supplemented with $CuSO_4$ as indicated.

Expression of Green Fluorescent Reporter Protein in Isolated Arabidopsis protoplasts. Messenger RNA isolation from 14-day old seedlings and cDNA synthesis are described [22]. Primers to amplify the cDNA containing the coding sequence for *AtCCS* (AGI: At1g12520) by PCR are as follows

(upstream/downstream): 5' TACCGCGGTACCATGGCG ACTGCTCT and 5' ATCCGCGGATCCTTAACCCTTACTGGCCAGGAAA. The PCR fragments were subcloned in *Kpn*1/*Bam*H1 digested pBluescript-SK (Stratagene, San Diego CA). To generate a GFP fusion, the full coding sequence of *AtCCS* was amplified to introduce *Sal*1/*Nco*1 restriction sites for cloning in the GFP reporter plasmid 35□-SGFP(S65T) [23], generously provided by Dr. Norbert Rolland (Université Joseph Fourier, France). Enzymes, cellulase Onozuka R-10 and Macerozyme R-10 were obtained from Karlan Research Products (Santa Rosa, CA, USA). Protoplasts were isolated and transformed as described [24]. A confocal laser-scanning microscope (FVX-IHRT Fluoview Confocal LSM, Olympus, Melville, NY, USA) with Kr/Ar laser excitation (488 nm) was used to monitor green fluorescence (530 nm) and red chlorophyll auto fluorescence (660 nm). Images were captured and processed using Fluoview software at a 90X magnification at a scan speed of 0.45 seconds for 256X256 pixel area. Scan slices were 1.0 μm thick.

Yeast complementation. The *AtCCS* mature sequence was cloned under the control of the constitutive PGK promoter in the yeast shuttle vector pFL61 [25] after PCR amplification to introduce *Not*1 restriction sites. The yeast *lys7* mutant [13] was obtained from Dr. V. Culotta (Johns Hopkins University). Strains were transformed using the Li-acetate method; transformed colonies were selected on SC minimal media lacking uracil. Complementation was tested by plating serial dilutions on SC-minimal media lacking lysine [13] or

supplemented with Menadione (sigma, St Louis, Mo). Plates were incubated at 30°C for 3 days.

Miscellaneous methods. Protein was assayed according to [26]. SOD activity assays were performed as described [8]. RNA blots were performed as described [22]. DNA constructs were sequenced in two directions at Davis Sequencing (Davis, CA, USA). Multiple protein alignments were performed with the ClustalW program [27].

3. Results

In analogy to the situation in yeast, Cu chaperones may exist, which deliver Cu to specific targets within the chloroplast. Although the *Arabidopsis* genome encodes many proteins with Cys-x-x-Cys containing possible heavy metal binding domains, our database searches revealed the presence of just 2 candidate genes encoding putative soluble proteins with a heavy metal binding domain and a putative chloroplast targeting sequence (AGI nrs: At1g12520 and At2g28660). Of these 2 genes, one (At1g12520) encodes a protein similar to Ccs1p/Lys7p, the yeast Cu-chaperone for Cu/ZnSOD. Earlier database searches suggested that *AtCCS* may be the only *LYS7*-like sequence in *Arabidopsis* and it has been speculated that the gene encodes gene products in both plastids and cytosol [16]. The same protein had been identified as a potential homologue of Ccs1p/Lys7p, however that clone lacked a significant portion of the N-terminal sequence [17]. We used RT-PCR to obtain the full-length protein-coding sequence for *AtCCS*. Sequence comparison of the

cloned cDNA and the published genomic sequence (TAIR) indicated the presence of 6 exons and 5 introns (Figure 1A), an organization that is shared with the potato homologue [18]. The cDNA sequence confirmed the exon assignment in the MIPS database (http://mips.gsf.de/proj/thal/db/) and indicates that it encodes a protein of 320 amino acids. A sequence alignment of AtCCS with other predicted CCS proteins is shown in Figure 1B. The N-terminal region of AtCCS includes a predicted 66-amino acid cleavable chloroplast targeting sequence, and similar sequences are found in the other plant CCS proteins. The predicted mature AtCCS protein shares a three-domain structure typical of CCS proteins, including a conserved Atx1-like domain with canonical MxCxxC binding motifs (domain I), a central domain with similarity to a portion of Cu/ZnSOD (domain II) and a C-terminal region which includes two conserved cysteines (domain III).

To investigate if AtCCS is a functional homologue of yeast Ccs1p/Lys7p the *Arabidopsis* protein was expressed without its transit sequence in a yeast *lys7* mutant. We tested for functional complementation by assaying the growth phenotype on media that lack lysine or contain the superoxide generator menadione (Figure 2A) and by assaying SOD activity (Figure 2B). An isogenic wild-type strain and a *lys7* mutant transformed with an empty vector were used as controls. Expression of AtCCS rescues both the growth defect phenotype and SOD activity in the *lys7* mutant. We conclude that AtCCS is functional as a copper chaperone for SOD in yeast.

In order to investigate the intracellular localization of AtCCS, the coding sequence for full-length AtCCS protein was fused to the coding sequence for

green fluorescent protein in a transient expression vector under control of the Cauliflower Mosaic Virus 35S (CAMV35S) constitutive promoter. Isolated *Arabidopsis* protoplasts were transformed with this plasmid and analyzed for GFP expression. Protoplasts transformed with control CAMV35S:GFP exhibited GFP fluorescence in the cytosol as shown in Figure 3. In contrast, AtCCS:GFP is localized in structures corresponding to chloroplasts when compared to red chlorophyll fluorescence, indicating that AtCCS is imported along with the GFP passenger into chloroplasts.

We analyzed the expression of *AtCCS* using RNA blots which indicated that *AtCCS* is expressed both in root and shoot tissues (Figure 4A). A striking increase in *AtCCS* mRNA levels was seen in shoots during natural senescence (Figure 4B). To investigate the effects of Cu feeding and plastid Cu levels we compared the mRNA expression in shoot tissue of WT and *paa1* and *paa2* mutant plants under different conditions of Cu supply (Figure 4C). *AtCCS* mRNA levels are clearly induced by elevated Cu levels (Figure 4C). Interestingly, in the *paa2* mutant which accumulates Cu in the stroma [9], *AtCCS* mRNA levels were induced at much lower Cu concentrations compared to the WT and *paa1* mutant. RT-PCR experiments showed that treatment with other metal ions such as Fe did not induce *AtCCS* expression.

4. Discussion

The protein encoded by At1g12520 (*AtCCS*), is most likely the metallo-chaperone that delivers Cu to CSD2 in the chloroplast stroma, in view of its sequence, the complementation data and the observed localization in plant

cells. However, *AtCCS* is the only candidate in the *Arabidopsis* genome with high conservation as a copper chaperone for superoxide dismutase. It is therefore possible that AtCCS delivers copper to both CSD1 and CSD2, perhaps utilizing an alternative translation start site that skips the chloroplast targeting peptide as was suggested based on bioinformatic data [16]. The expression of chloroplastic and CSD2 as well as cytosolic CSD1 is dramatically up-regulated by Cu [9] and AtCCS is co-regulated with the CSD1 and CSD2 targets indicating an important role of delivery Cu in the regulation of oxidative stress protection. Interestingly and consistent with our findings, *AtCCS*, *CSD1* and *CSD2* were found to be down-regulated together in response to both Zn and Cu deficiency in a transcript profiling study using micro-arrays [28]. The regulation of *AtCCS* in response to Cu and senescence may also reflect the need for protection from oxidative stress and the need to buffer excess Cu.

It is of interest to compare the reported phenotypes of plants that are deficient in stromal Cu/ZnSOD [29] and plastocyanin [30]. *Arabidopsis* expresses two plastocyanin genes, that are closely related in sequence [31,32]. Silencing of both copies of plastocyanin leads to a very severe growth phenotype [33] and plants with insertions in both plastocyanin genes cannot be maintained on soil [30]. Thus, in higher plants, plastocyanin is an essential protein and it can be expected that Cu delivery to plastocyanin is a priority for plants. *Arabidopsis* knock-down mutants for *csd2* were reported to display a very severe and light-dependent growth phenotype [29]. Photo-reduction of O_2 at PSI is thought to be an important mechanism of superoxide ion formation

[1]. In *paa1*, the reduced electron transport rate may diminish the need for photo-protection via an active water-water cycle because photosystem-I will be more oxidized [8]. We hypothesize that for optimal photosynthesis, the chloroplast needs to have a Cu delivery system that balances the activity of lumenal plastocyanin and stromal SOD enzymes under variable metal supply, ensuring that sufficient SOD activity is present to prevent oxidative damage if plastocyanin is present and PSI can be reduced (see Figure 5). The available data suggest that this is achieved by a reciprocal regulation of FeSOD and CSD2 expression [9] and regulation of the copper chaperone for SOD in response to Cu in the chloroplast (Figure 4C). On low Cu media WT plants still produce active plastocyanin, whereas SOD activity is provided by FeSOD alone. The reduced activity of CSD2 under these conditions may help save Cu for delivery to plastocyanin in the lumen. Under Cu sufficient conditions *CSD2* is transcribed and a balanced delivery of Cu to stromal CSD2 (likely via AtCCS) and to plastocyanin must take place. Under high Cu conditions in the plastid both plastocyanin and in particular stromal Cu/ZnSOD may help to buffer Cu concentrations.

We observed the switching between a Cu-enzyme (CSD2) and an Fe-enzyme (FeSOD) in chloroplasts in response to nutrient status. A Cu/ZnSOD is not found in cyanobacteria or in the eukaryotic green algae *Chlamydomonas rheinhardtii*, which depend on FeSOD activities and therefore this switching does not occur in these organisms. Plastocyanin is indispensable in plants [30] and therefore a priority for Cu delivery. However in many algae, including *Chlamydomonas* a cytochrome-*c* [6] can functionally replace plastocyanin

under low Cu conditions [34]. This presumably saves Cu for other essential functions such as respiration, which take priority under Cu starvation. Thus, Cu delivery pathways in higher plants and algae may have adapted differently to ensure delivery of Cu to the most essential Cu proteins in each organism.

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References

[1] Asada, K. (1999). THE WATER-WATER CYCLE IN CHLOROPLASTS: Scavenging of Active Oxygens and Dissipation of Excess Photons. Annu Rev Plant Physiol Plant Mol Biol 50, 601-639.

[2] Bowler, C. (1992). Superoxide Dismutase and Stress Tolerance. Annual Review of Plant Physiology and Plant Molecular Biology 43, 83.

[3] Kliebenstein, D.J., Monde, R.A. and Last, R.L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118, 637-50.

[4] Nelson, N. (1999). Metal ion transporters and homeostasis. Embo J 18, 4361-71.

[5] Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D. and Van Montagu, M. (1995). Molecular characterization of a putative Arabidopsis thaliana copper transporter and its yeast homologue. J Biol Chem 270, 28479-86.

[6] Sancenon, V., Puig, S., Mira, H., Thiele, D.J. and Penarrubia, L. (2003). Identification of a copper transporter family in Arabidopsis thaliana. Plant Mol Biol 51, 577-87.

[7] Sancenon, V., Puig, S., Mateu-Andres, I., Dorcey, E., Thiele, D.J. and Penarrubia, L. (2004). The Arabidopsis copper transporter COPT1 functions in root elongation and pollen development. J Biol Chem 279, 15348-55.

[8] Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K.K. and Pilon, M. (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15, 1333-46.

[9] Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M. and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17, 1233-51.

[10] Harrison, M.D., Jones, C.E., Solioz, M. and Dameron, C.T. (2000). Intracellular copper routing: the role of copper chaperones. Trends Biochem Sci 25, 29-32.

[11] Lamb, A.L., Torres, A.S., O'Halloran, T.V. and Rosenzweig, A.C. (2001). Heterodimeric structure of superoxide dismutase in complex with its metallochaperone. Nat Struct Biol 8, 751-5.

[12] Rae, T.D., Torres, A.S., Pufahl, R.A. and O'Halloran, T.V. (2001). Mechanism of Cu,Zn-superoxide dismutase activation by the human metallochaperone hCCS. J Biol Chem 276, 5166-76.

[13] Culotta, V.C., Klomp, L.W., Strain, J., Casareno, R.L., Krems, B. and Gitlin, J.D. (1997). The copper chaperone for superoxide dismutase. J Biol Chem 272, 23469-72.

[14] Brown, N.M., Torres, A.S., Doan, P.E. and O'Halloran, T.V. (2004). Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu,Zn superoxide dismutase. Proc Natl Acad Sci U S A 101, 5518-23.

[15] Furukawa, Y., Torres, A.S. and O'Halloran, T.V. (2004). Oxygen-induced maturation of SOD1: a key role for disulfide formation by the copper chaperone CCS. Embo J 23, 2872-81.
[16] Wintz, H. and Vulpe, C. (2002). Plant copper chaperones. Biochem Soc Trans 30, 732-5.
[17] Zhu, H., Shipp, E., Sanchez, R.J., Liba, A., Stine, J.E., Hart, P.J., Gralla, E.B., Nersissian, A.M., and Valentine, J.S. (2000). Cobalt(2+) binding to human and tomato copper chaperone for superoxide dismutase: implications for the metal ion transfer mechanism. Biochemistry 39, 5413-21.
[18] Trindade, L.M., Horvath, B.M., Bergervoet, M.J. and Visser, R.G. (2003). Isolation of a gene encoding a copper chaperone for the copper/zinc superoxide dismutase and characterization of its promoter in potato. Plant Physiol 133, 618-29.
[19] Ruzsa, S.M. and Scandalios, J.G. (2003). Altered Cu metabolism and differential transcription of Cu/ZnSod genes in a Cu/ZnSOD-deficient mutant of maize: evidence for a Cu-responsive transcription factor. Biochemistry 42, 1508-16.
[20] Tsang, E.W., Bowler, C., Herouart, D., Van Camp, W., Villarroel, R., Genetello, C., Van Montagu, M. and Inze, D. (1991). Differential regulation of superoxide dismutases in plants exposed to environmental stress. Plant Cell 3, 783-92.
[21] Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.
[22] Burkhead, J.L., Abdel-Ghany, S.E., Morrill, J.M., Pilon-Smits, E.A. and Pilon, M. (2003). The Arabidopsis thaliana CUTA gene encodes an evolutionarily conserved copper binding chloroplast protein. Plant J 34, 856-67.
[23] Miras, S., Salvi, D., Ferro, M., Grunwald, D., Garin, J., Joyard, J. and Rolland, N. (2002). Non-canonical transit peptide for import into the chloroplast. J Biol Chem 277, 47770-8.
[24] Sheen, J. (2001). Signal transduction in maize and Arabidopsis mesophyll protoplasts. Plant Physiol 127, 1466-75.
[25] Minet, M., Dufour, M.E. and Lacroute, F. (1992). Complementation of Saccharomyces cerevisiae auxotrophic mutants by Arabidopsis thaliana cDNAs. Plant J 2, 417-22.
[26] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-54.
[27] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-80.
[28] Wintz, H., Fox, T., Wu, Y.Y., Feng, V., Chen, W., Chang, H.S., Zhu, T. and Vulpe, C. (2003). Expression profiles of Arabidopsis thaliana in

mineral deficiencies reveal novel transporters involved in metal homeostasis. J Biol Chem 278, 47644-53.
[29] Rizhsky, L., Liang, H. and Mittler, R. (2003). The water-water cycle is essential for chloroplast protection in the absence of stress. J Biol Chem 278, 38921-5.
[30] Weigel, M., Varotto, C., Pesaresi, P., Finazzi, G., Rappaport, F., Salamini, F. and Leister, D. (2003). Plastocyanin is indispensable for photosynthetic electron flow in Arabidopsis thaliana. J Biol Chem 278, 31286-9.
[31] Vorst, O., Oosterhoff-Teertstra, R., Vankan, P., Smeekens, S. and Weisbeek, P. (1988). Plastocyanin of Arabidopsis thaliana; isolation and characterization of the gene and chloroplast import of the precursor protein. Gene 65, 59-69.
[32] Schubert, M., Petersson, U.A., Haas, B.J., Funk, C., Schroder, W.P. and Kieselbach, T. (2002). Proteome map of the chloroplast lumen of Arabidopsis thaliana. J Biol Chem 277, 8354-65.
[33] Gupta, R., He, Z. and Luan, S. (2002). Functional relationship of cytochrome c(6) and plastocyanin in Arabidopsis. Nature 417, 567-71.
[34] Merchant, S. (1998) Synthesis of Metalloproteins Involved in Photosynthesis: Plastocyanin and Cytochromes. In The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas. (Rochaix, J.-D., Goldshmidt-Clermont, M. and Merchant, S., ed.^eds), pp. 597-611. Kluwer Academic Publishers Boston.

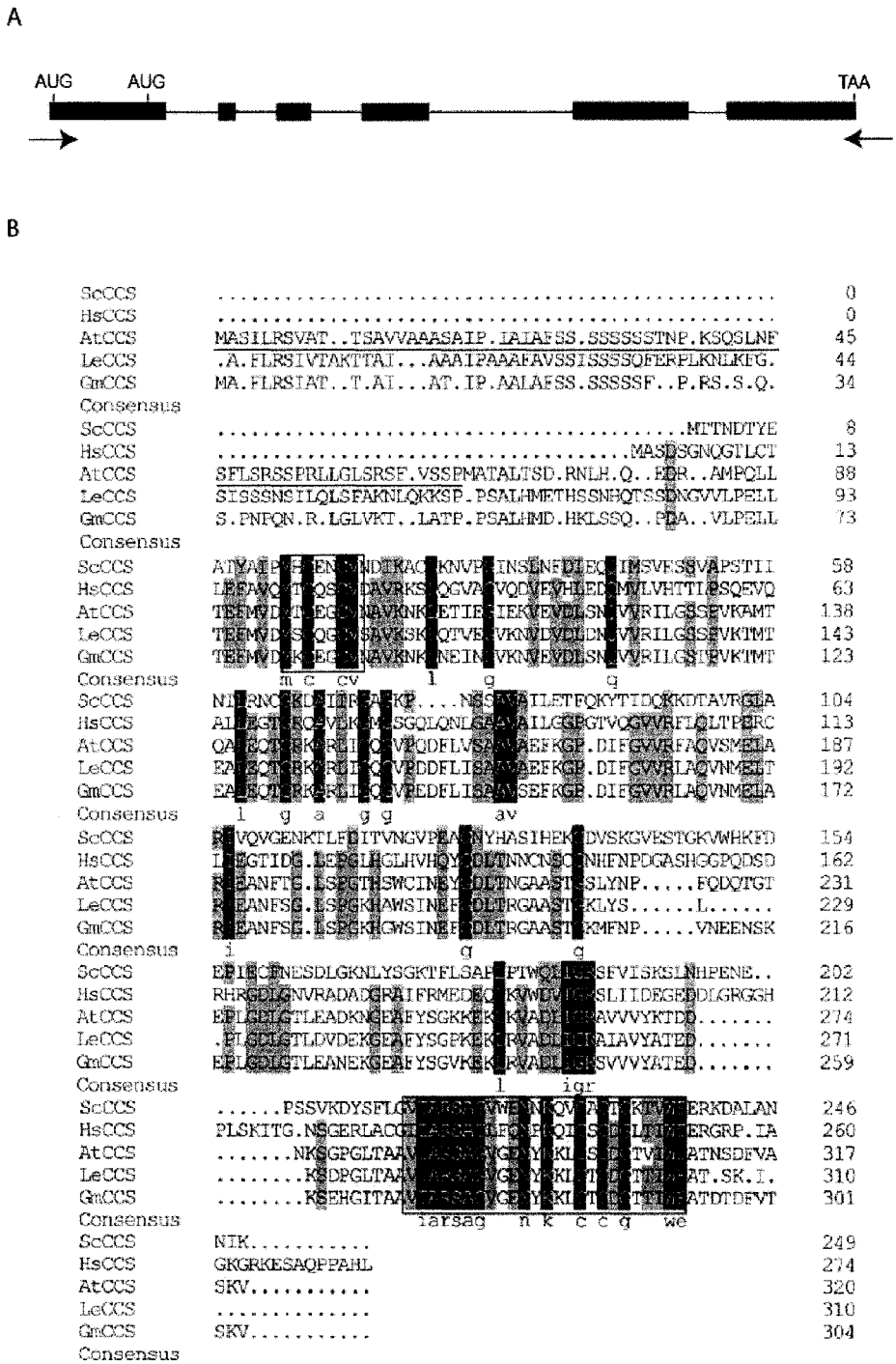

figure 1

Figure 1. Sequence alignment and domain structure of *Arabidopsis* CpCCS. Panel A: Genomic structure of AtCCS. Exons are indicated by solid boxes, introns by lines. Primers used to amplify *AtCCS* cDNA are indicated by arrows. Panel B: alignment of the CCS from *Arabidopsis* (AtCCS), yeast (ScCCS), humans (HsCCS), tomato (LeCCS) and maize (GmCCS). The predicted chloroplast transit sequence of AtCCS is underlined. Conserved regions implied in metal binding are indicated by boxes.

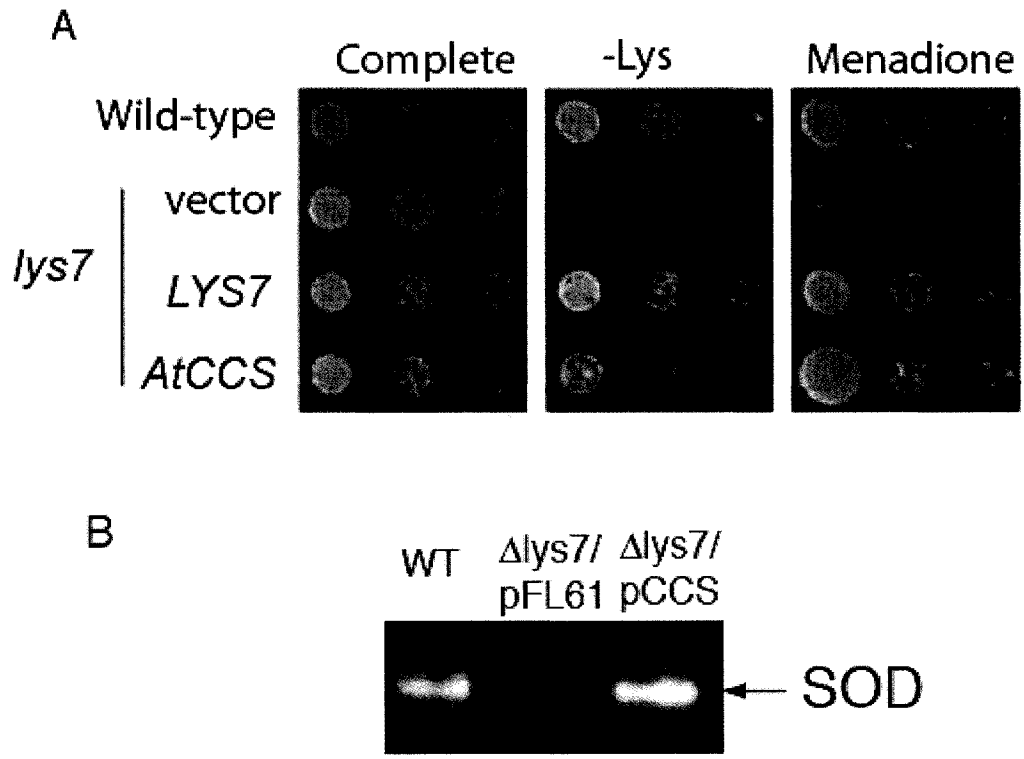

Figure 2. Functional complementation of the yeast *lys7/ccs1* mutant by *AtCCS*. Panel A: Complementation of the growth phenotype of *lys7*. *S. cerevisiae* wild type and *lys7* mutant cells transformed with empty plasmid (vector), or vector containing yeast *LYS7/CCS1* or mature *AtCCS* were assayed for growth on SD (Complete), SD without lysine (-Lys), and YPD with 25 μM menadione. Cells were grown in SD lacking uracil to exponential phase (~A_{600} = 1.0), spotted in 10-fold serial dilutions starting at A_{600}= 0.1 and incubated at 30°C for 3 days. Panel B: Native gel assay for Cu/ZnSOD activity in yeast. Wild-type or mutant cells transformed with the indicated plasmids were grown in SD media and cell extracts (10 μg protein) tested for SOD activity.

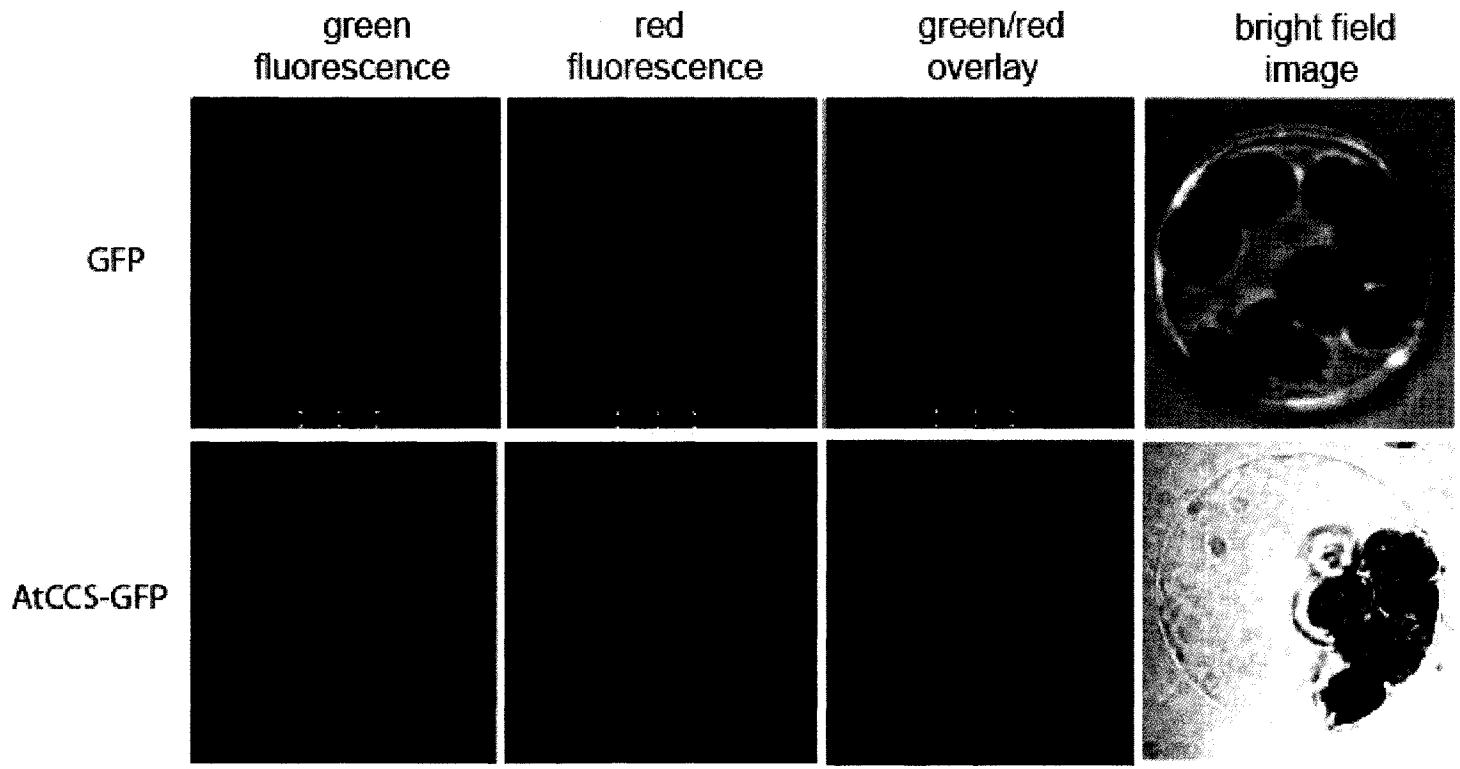

Figure 3. CpCCS is localized in chloroplasts. The coding sequence of the AtCCS precursor was fused to GFP and expressed in protoplasts. Plasmid expressing GFP alone was used as a control. Cells were analyzed 16 h after transformation by confocal microscopy.

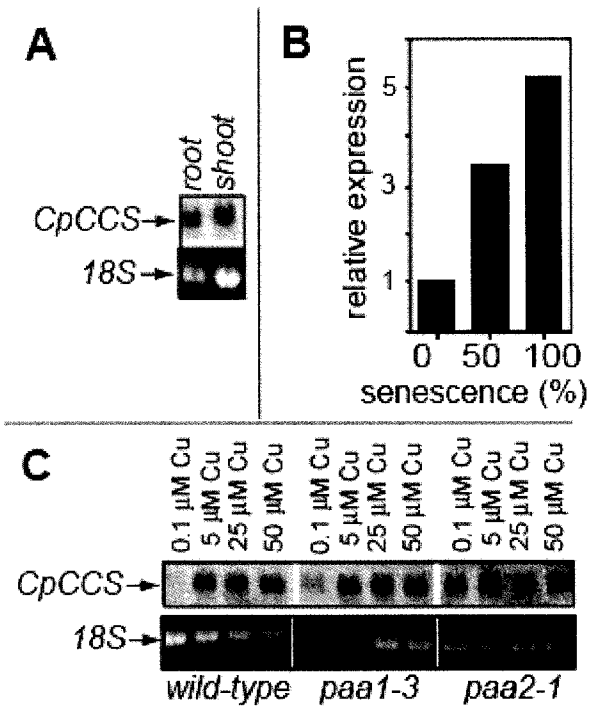

Figure 4. mRNA expression analysis of CpCSS. Panel A: Expression in roots and shoots. Panel B: *AtCCS* expression during senescence. RNA was prepared from the leaves of adult plants. CCS expression was analyzed at different senescence stages, indicated by the percentage of yellow leaf surface. Panel C: Expression in shoots in response to Cu feeding and in response to mutations in the chloroplast Cu transporters *paa1 and paa2*. Equal amounts of total RNA were loaded and separated on agarose gels, blotted to Hybond membranes and probed with a AtCCS probe. All experiments were performed in duplicate. Quantitative data are the average of two measurements normalized to the control.

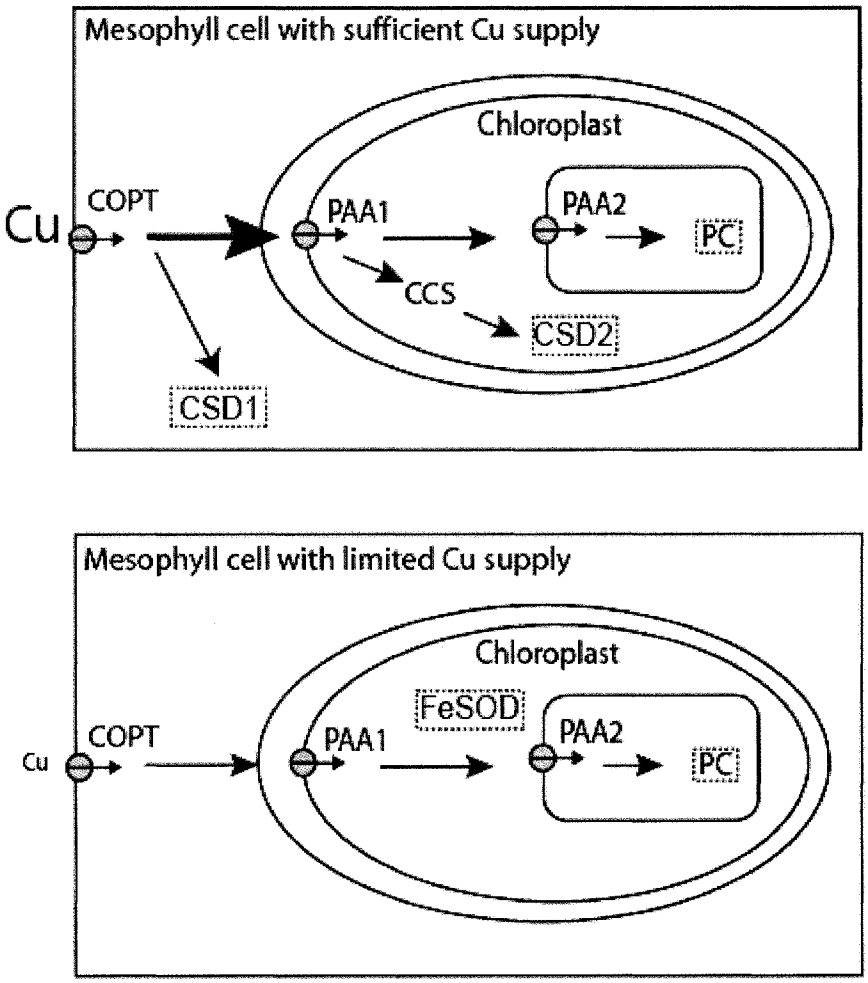

Figure 5. A model for SOD regulation and Cu delivery in chloroplasts. Cu enters the chloroplast by the PAA1 transporter. When Cu supply is sufficiently high, *CSD1* and *CSD2* and the Cu-chaperone gene *CCS* are transcribed. The CSD2 protein functions to detoxify the superoxide produced as a result of photosynthetic electron transport. Under these conditions PAA2 can deliver Cu to plastocyanin and both CSD1 and CSD2 may help to absorb excess available Cu and prevent toxicity. When Cu supply is limited, the mRNA and protein levels for CSD1, CSD2 and CCS are reduced while the FeSOD mRNA and protein become abundant. This regulation allows PAA2 to effectively deliver Cu to plastocyanin without the need to compete with CCS for Cu under these limiting conditions.

CHAPTER 4

Effects of altered CCS expression on copper homeostasis in *Arabidopsis* with a novel interaction between CCS and HMA5(N)

Abstract

Copper is an essential micronutrient for plants and plays a crucial role in processes such as photosynthesis and reactive oxidative species detoxification. *Arabidopsis* contains three isoforms of Cu,Zn superoxide dismutase (SOD) enzymes and the copper chaperone for SOD (CCS) is required for copper delivery to Cu,ZnSODs within the plant cell. All types of SODs have the important function of reducing reactive oxygen species (ROS), which can be a byproduct of photosynthesis and respiration. The chloroplast contains two different isoforms of SODs that require iron or copper and zinc transport across the envelope membrane for enzyme activity where metal cofactor availability may play a role in regulation. Oxidative stress and nutrient demands can vary based on tissue type and the developmental stage of the organism. In this investigation, we study how altered CCS expression affects protein expression of superoxide dismutase enzymes at various copper concentrations and at different developmental stages. The CCS expression levels are influenced by copper concentrations. Furthermore, we describe a

novel interaction between the CCS copper chaperone and the amino terminal domain of the HMA5 transporter and propose a new model for copper transport in the plant cell.

Abbreviations used:

CaMV35S, Cauliflower Mosaic Virus 35S promoter; CCS, copper chaperone for Cu/Zn superoxide dismutase; Cu,ZnSOD, Cu,Zn superoxide dismutase; CSD1, cytosolic Cu,Zn SOD; CSD2, stromal Cu,Zn SOD; FeSOD, Fe superoxide dismutase; F_v/F_m, the quantum efficiency of open photosystem II centers; MnSOD, Mn superoxide dismutase; NPQ, Non-photochemical quenching; PC, plastocyanin; PSII, Photosystem II; ΦPSII, Quantum yield of photosystem II photochemistry; qP, Photochemical quenching; SOD, superoxide dismutase.

1. Introduction

Arabidopsis thaliana contains a total of seven superoxide dismutase genes, which encode three isoforms of SOD enzymes [1]. These enzymes are categorized by their metal cofactor, iron, manganese, or copper and zinc [2]. All types of SODs have the important function of reducing reactive oxygen species (ROS), which can be a byproduct of cellular processes such as photosynthesis and respiration. Superoxide dismutases are responsible for the conversion of superoxides (O_2^-) to hydrogen peroxide (H_2O_2) which prevents the formation of highly reactive hydroxyl radicals (OH). Catalases and peroxidases can then reduce hydrogen peroxide to water [1,3].

In *Arabidopsis* the major SOD isoforms are found in several intracellular locations: a cytosolic Cu,ZnSOD (CSD1), a chloroplastic Cu,ZnSOD (CSD2), and peroxisomal Cu,ZnSOD (CSD3), as well as a mitochondrial MnSOD (MSD1) and chloroplastic FeSOD (FSD1) [1]. The chloroplast contains two different isoforms of SODs that require iron or copper and zinc transport across the envelope membranes for enzyme activity. Due to other copper requirements within the chloroplast essential for photosynthesis, it has been suggested that the regulation of FeSOD and CSD2 is controlled by metal availability, particularly copper [4,5]. Under low concentrations of copper, priority is for delivery to plastocyanin in the chloroplast and as a result there is expression and activity of FeSOD. However, when copper supplies are sufficient the metal is delivered to CSD2 for activity.

Recently, it has been proposed that the regulation of some SODs, including *CSD1* and *CSD2* are controlled by microRNAs. MicroRNAs are small fragments of RNA (approximately 20 nucleotides long) that regulate target genes through sequence-specific degradation of mRNA sequences [6]. The microRNA family that has been identified for target degradation of *CSD1* and *CSD2* is *miR398* [7,8]. Experimental data has demonstrated that *miR398* responds to both environmental stress as well as copper concentrations to regulate the expression of both genes [5,9]. It has been hypothesized that *miR398* is part of a regulatory mechanism that detects copper availability needed for proper function of CSD1 and CSD2 and regulates the transcripts of those genes accordingly [5].

Copper homeostasis is tightly regulated in plant cells, which is most likely due to the potential toxicity of free metal ions. However, copper is an essential micronutrient that is required for photosynthesis, respiration, ethylene response, as well as superoxide dismutase activity. Copper enters the plant cell through a Ctr-like transporter, COPT1 [10], and is shuttled to one of its many targets via copper chaperones. Several possible chaperones have already been identified in *Arabidopsis*, including ATX1, CCH, and CCS [11]. Both ATX1 and CCH proteins are functional homologs of the yeast ATX1 [12,13]. These cytosolic metallochaperones have been shown to interact with the RAN1 transporter in the endoplasmic reticulum or Golgi membrane [12] and HMA5 in the plasma membrane [14]. The RAN1 (Responsive-to-antagonist1) transporter is a homolog of the yeast Ccc2 protein and functions to translocate copper into the endomembrane system were it is required for ethylene signaling [15,16]. The HMA5 membrane transporter is highly expressed in roots and flowers, however based on mutant analysis the function seems to be detoxification of copper within root tissue [14].

Arabidopsis contains one *CCS* gene (At1g12520) which is expressed in root and shoot tissue and increases expression levels during senescence [4]. The CCS protein, a functional homolog of the yeast CCS, is co-localized to both the cytosol and the stroma and is responsible for delivering copper to Cu,ZnSODs [4,17]. Cystallographic structure analysis of CCS proteins from yeast and humans indicate that it consists of three domains: an ATX-like domain (domain I), a SOD-like domain (domain II), and a unique domain

(domain III) [18-20]. The CCS chaperone also contains a CxxC metal binding sequence in the amino terminal, ATX-like domain [19].

Plants require the transport of micronutrients over long distances and across several membranes for proper delivery to target enzymes. Additionally, demands for nutrients and the production of reactive oxygen species can vary based on tissue type and the developmental stage of the organism. Here, we investigate the effects of altering CCS protein expression on other proteins involved in copper homeostasis and superoxide dismutase. We describe a growth rate and chlorophyll fluorescence phenotype of *CCS* overexpressing plants compared to a *CCS* knockout mutant. We report a change in expression levels of CCS and SOD proteins at different developmental stages in deficient and sufficient copper concentrations. Furthermore, we describe a novel interaction between the CCS copper chaperone and the amino terminal domain of the HMA5 transporter.

2. Materials and Methods

Plant material and growth conditions. The *Arabidopsis CCS* T-DNA insert plant line was obtained from the SALK institute (Salk_025986). Mutant plants were backcrossed to wildtype three consecutive times and homozygous seed was collected. The *hma5-1* plant line was obtained from Lola Peñarrubia (Departament de Bioquímica i Biologia Molecular, Universitat de València, Spain) and has been described [14]. *Arabidopsis* plants were grown on ½ Murashige and Skoog (MS) tissue culture media containing 1% sucrose and 0.4% agarose. Treatments were supplemented with either $CuSO_4$ or methyl

viologen (MV) to the growth media as stated [21]. Plants were grown for 7-35 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.

Overexpression of CCS in Arabidopsis. Amplification of DNA encoding the full length sequence of CCS including precursor was performed by PCR using 5'-catgccatggatggcatcaattctcaggtcagt-3' (forward) and 5'-cgcggattcttaaaccttactggccacgaaat-3' (reverse) primers. cDNA from wildtype *Arabidopsis* plants was used as the template for fragment amplification. The CCS coding region was then cloned in frame into the pFGC5941 vector containing the *CaMV35S* promoter and sequenced in two directions using the gene specific primers. *Arabidopsis thaliana* (Col-0) plants were transformed with the pFGC5941-*CCS* vector via *Agrobacterium* using the floral dip method [22]. Glufosinate ammonium (AgroEvo, Inc.) resistance (0.25 mg/mL) was used to screen soil grown plants for selection of the plasmid. Individuals from two separate plant lines (*OX11* and *OX14*) were selected, grown for two generations and homozygous seed was collected.

Protein Analysis by Immunoblotting and Native Assays. Soluble protein extraction of plant tissue for SDS-PAGE and NATIVE-PAGE analysis was performed according to [23]. Protein was quantified using the Bradford method [24] and bovine serum albumin was used for a standard curve. A total of 10 μg of protein extract was fractionated on PAGE gels for SOD activity analysis or immunodetection. Activity of SODs was determined by staining NATIVE-PAGE

gels according to [25,26] and 10-20% Tris-HCL gradient gels (Bio-Rad Laboratories) were used to resolve CSD1 and CSD2 activity. The full length AtCCS recombinant protein was expressed in *E. coli* and purified. A polyclonal antibody was then raised in rabbits against the CCS purified protein (Pocono Rabbit Farm and Laboratory Inc, PA, USA). Other antibodies used for immunodetection were obtained from D. Kliebenstein (University of California, Davis, CA) [1] or have been described [26]. All antibodies were raised against *Arabidopsis* purified proteins with the exception of plastocyanin which was raised against spinach.

Chlorophyll Fluorescence Measurements. Plants were grown in one half MS medium with 1% agarose for 14 days in 12/12hr light/dark cycles at a light intensity of 120 µmol photons $m^{-2} \cdot s^{-1}$ and 23°C. Chlorophyll fluorescence was imaged using a Fluorcam 700MF, controlled by v. 5.0 PSI Fluorcam software (Photon System Instruments, Brno, Czech Republic), on one hour dark-adapted plants before and after high light treatment. Fluorescence was recorded for plants grown at low light intensity. Plants were then placed in continuous high light (500 µmol photons $m^{-2} \cdot s^{-1}$) and a temperature of 25°C for 64 hours. Immediately after high light treatment plants were measured for chlorophyll fluorescence again as described above.

Bacteria and yeast cell strains. Escherichia coli strain DH5α (F^- φ80*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) was cultured in Luria-Bertani (LB) broth Miller (EMD Biosciences)

medium with the appropriate antibiotic and was used for cloning and propagation of plasmids. *Saccharomyces cerevisiae* strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3, MEL1GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2, URA3::MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ*) [27] was obtained from Clontech Laboratories, Inc. (Mountain View, CA, USA) and was cultured in yeast extract/peptone/dextrose (YPD) (1% (w/v) yeast extract (Fisher Scientific), 2% (w/v) peptone (Fisher Scientific), 2% (w/v) D-glucose (Fisher Scientific)) medium or synthetic complete drop out (SD) medium (Difco) lacking tryptophan and leucine for selection and maintenance of plasmids.

Plasmid constructs for yeast two-hybrid system. The pGBK-HMA5(N), pGAD-ATX1, pGAD-CCH, and pGAD-CCHΔ plasmids were obtained from Sergi Puig (Departament de Bioquímica i Biologia Molecular, Universitat de València, Spain) and are described in the publication [14]. Amplification of DNA encoding the full length sequence of CCS was performed by PCR using Expand High Fidelity polymerase (Roche). Oligonucleotide primer sequences used to generate DNA fragment is 5'-gggaattccatatggcgactgctctcacttc -3' (forward) and 5'-ataggaatgcggccgcggatccttaaaccttactggccacgaaa-3' (reverse). Genomic DNA isolated from wildtype *Arabidopsis* plants was used as a template for fragment amplification. The coding region of CCS was cloned in frame into pGBKT7 and/or pGADT7 vectors (Matchmaker™ Yeast Two-Hybrid System 3, Clontech) using the *Nde*I and *Bam*HI restriction sites.

Yeast two-hybrid spot assay. Yeast cells were simultaneously co-transformed with a plasmid containing an activation domain fusion and one containing a binding domain fusion using the lithium acetate (LiAc) method [28]. Selection of the transformants was performed on 1.5% agar plates lacking tryptophan and leucine and several individual colonies were purified for spot assays. Liquid cultures of yeast co-transformed with pGAD and pGBK based vectors were propagated in the proper medium for selection of both plasmids for 24 hours at 30° C, shaking at 260 RPM. The optical density of yeast cultures were measured at 600nm using a DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter, Inc.) and diluted to 0.1 OD_{600}, then further diluted to 0.01 OD_{600} for spot assays. An 8-channel pipettor (VWR International) was used to transfer 3 µL of each dilution onto three separate agar plates containing SD minimal medium lacking tryptophan, leucine or medium lacking tryptophan, leucine, histidine, and adenine or medium lacking tryptophan, leucine, histidine, and adenine supplemented with 2.5 mM 3-amino-1,2,4-triazole (Sigma). Agar plates were then incubated at 30° C for five days and each assay was repeated in at least three independent experiments.

β-galactosidase activity assays. The procedure for quantitative estimates of yeast two-hybrid interactions using β-galactosidase was modified from the Yeast Protocol Handbook (Clontech) [28]. Liquid cultures of yeast co-transformed with cloned pGAD and pGBK vectors were propagated in the proper dropout medium for selection of both plasmids and supplemented with bathocuproine disulfonic acid (BCS) (Sigma-Aldrich) for 48 hours at 30° C,

shaking at 260 RPM. Control cultures were propagated in the absence of BCS. Liquid β-galactosidase activity assays were then carried out following the Clontech protocol using chlorophenol red-β-D-galactopyranoside (CPRG) (Calbiochem) as the substrate [28]. Each culture was measured in triplicate for an individual experiment and three independent experiments were performed. β-galactosidase Miller units [29,30] were calculated and quantities are presented as average values ± mean standard deviations for the three experiments combined.

Sequence and statistical analysis. Gene and protein information was obtained through The Arabidopsis Information Resource (TAIR) database. Sequence alignments were performed using the CLUSTAL W (1.83) multiple sequence alignment tool [31]. A model of the HMA5 amino-terminal domain was generated using the SWISS-MODEL Server and DeepView - Swiss-PdbViewer Software [32-35]. Plasmid DNA was sequenced in two directions using the forward and reverse gene specific primers used in cloning and/or the forward and reverse plasmid specific primers. Sequencing was performed by Macromolecular Resources (Colorado State University, Fort Collins, CO, USA). Statistical analysis was performed using the Jump-in software package (SAS Institute, Cory, NC).

3. Results

The *Arabidopsis CCS* gene (At1g12520) contains six exons with two separate ATG sites located in the first exon (Figure 1). We obtained a

knockout mutant plant line for the *CCS* gene (Salk_025986). Knockout plants grown on soil did not express CCS protein and also lost the activity of both CSD1 and CSD2 compared to wildtype plants. In addition to the knockout mutants, two separate overexpressing lines were produced to study the CCS protein. Overexpressors grown in limited copper supply (½ MS media) show a significantly elevated expression of CCS compared to wildtype plants (Figure 2).

CCS is an important protein involved in copper homeostasis and the superoxide dismutase enzymes that are targets for copper delivery are responsible for relieving oxidative stress within the plant cell. If the regulation of CCS is altered the growth rate of the plant may be affected as a result of the disturbance in copper homeostasis or oxidative stress. To investigate these possibilities, wildtype, *CCSKO*, *OX11*, and *OX14* plants were grown on ½ MS media and ½ MS media supplemented with $CuSO_4$ or with methyl viologen (MV) to induce oxidative stress. After 10 days of growth, primary root lengths were measured (Figure 3). Mean root length measurements were similar between all plant lines at low copper concentrations (Figure 4). However, at high levels of copper there was a significant difference between the overexpressing lines compared to the knockout mutant. In all treatments the knockout mutant had a decreased mean primary root length compared to wildtype. Additionally, in most cases *OX11* and *OX14* had an increased primary root length when compared to the same control plants with the most significant difference seen in the MV treatment.

Chlorophyll fluorescence has been widely used to study the effects of environmental stress to plant systems. Without the activity of several SODs as a result of a mutation in *CCS* oxidative stress may occur. Furthermore, the overexpression of the CCS protein may allow plants to cope more efficiently with any oxidative stress caused by the environment. Therefore, plant lines were grown in the presence of $CuSO_4$ or MV for 14 days and analyzed for chlorophyll fluorescence. These plants were then treated with continuous high light for 64 hours and chlorophyll fluorescence was measured again. This investigation would test how the mutant and overexpressing lines would manage the oxidative stress caused by high light under various copper concentrations. Before the high light treatment the mean values of chlorophyll fluorescence parameters (Fv/Fm, ΦPSII, qP, and NPQ) for wildtype, *CCSKO*, *OX11*, and *OX14* plants were similar (Table 1). However, after the high light treatment the *OX11* and *OX14* lines displayed a significantly different Fv/Fm measurement when compared to the *CCS* mutant plants. This indicates that the CCS overexpressers are able to manage oxidative stress more efficiently than the mutant and in some cases wildtype plants during copper feeding. This difference between plant lines is best illustrated in falsely colored images representing Fv/Fm values (Figure 5). In treatments containing 2 - 20 μM $CuSO_4$ CCS overexpressing plants had higher values of Fv/Fm as indicated by the dark orange or red color; whereas wildtype and knockout mutant plants appear yellow or green in color indicating a lower Fv/Fm value.

We wanted to investigate how changes in CCS expression affect other proteins involved in copper homeostasis. Wildtype, *CCSKO*, *OX11*, and *OX14*

plants were grown for 10 days on ½ MS media supplemented with $CuSO_4$ or MV for immunoblot and SOD activity analysis. Manganese SOD and HSP70 were used as loading controls for immunoblot assays (Figure 6). One half MS media containing 0.5 µM Cu was limited in copper supply, which can be detected by the presence and activity of FeSOD in all plant lines. The *CCS* knockout mutant showed no CCS expression in any treatment. Furthermore, there was little to no CSD1 or CSD2 expression and no activity of either SOD which was due to the defect in metal cofactor delivery. Interestingly, wildtype plants displayed an increased expression of CSD2 compared to overexpressing plants which was more apparent at low copper concentrations. However, CSD1 did not exhibit the same trend; rather, expression levels remained similar between *OX11*, *OX14*, and control plants. Interestingly, at low copper concentrations plants that have high CCS expression showed a decrease in CSD2 protein expression and enzyme activity. Additionally, an increase in copper concentrations resulted in a slightly higher expression of CCS protein that can be seen in both wildtype as well as the two overexpressors (Figure 7). Plastocyanin expression levels were not affected in any plant line across all treatments.

Copper requirements for proper growth vary in different types of plant tissue. In addition, oxidative stress can occur at different levels in various organs and at different stages of development. To investigate what copper protein expression patterns are seen as a result of developmental demands, wildtype plants were grown on ½ MS and ½ MS supplemented with 2 µM $CuSO_4$ for 35 days. Plant tissue was harvested and dissected every seven

days for immunoblot analysis. Interestingly, FeSOD was expressed only in photosynthetic tissue in ½ MS media, which may suggest copper sufficiency in root tissue and deficiency in shoot tissue (Figure 8). In copper supplemented media FeSOD was not expressed which indicates copper sufficiency in whole plant tissue. CSD1 expression is noticeably increased in root tissue compared to photosynthetic tissue; whereas the inverse is true for CSD2 expression. In limited copper concentrations CSD2 expression is higher in flowering tissue compared to leaf tissue. Expression of CCS was found at increased levels in photosynthetic tissue compared to root tissue in low and high copper treatments. In low copper, an increase in CCS expression was seen in inflorescent tissue compare to leaf or root tissue which was a similar trend to CSD2 expression. However, in sufficient copper CCS was equally expressed in leaf and inflorescence at 28 and 35 days.

CSD1 and CSD2 expression levels are slightly affected by altered CCS expression, which may suggest that CCS could acquire copper in the cytosol and stroma without delivery to SOD or CCS may transport the metal to additional targets. The amino terminal domain of HMA5 contains two copper binding motifs (MxCxxC) in two separate ATX-like domains (Figure 9). Protein-protein interactions have been demonstrated between HMA5(N) and ATX1 and CCH copper chaperones and since HMA5(N) contains two binding domains in two respective ATX-like domains it is possible for multiple chaperones to interact with the transporter. The yeast two-hybrid technique was utilized to investigate if CCS and HMA5(N) proteins can interact with each other. Interactions between HMA5(N) and ATX1 as well as CCHΔ were

observed in this investigation by spot assay as expected (Figure 10). In addition, a novel interaction between HMA5(N) and the full length version of CCS was detected. Quantitative estimates of protein interactions were obtained by β-galactosidase activity assays. Interactions with ATX1 and CCHΔ were approximately 2-fold higher than the empty vector control; whereas the interaction with CCS was 11-fold greater than the same control (Figure 11). When copper dependency was tested by the presence of BCS in the growth media none of the interactions were changed by the BCS addition. Interestingly, the HMA5-ATX1 interaction increased as copper availability decreased. This may be due to the addition of the fusion tag or the binding site for ATX1 is immediately adjacent to the metal ion when bound to HMA5(N). Interactions between CutA/CutA and KCBP(N)/KIPK were used as controls and are not believed to be copper dependent [36,37].

With this new protein-protein interaction between HMA5 and CCS, *hma5* mutant plants were studied in comparison to wildtype for protein expression analysis. Plants were grown in ½ MS media and ½ MS media supplemented with 10 μM $CuSO_4$ for 14 days and analyzed for immunoblot and SOD activity. In ½ MS media root and shoot tissue of wildtype and *hma5* plants had similar expression of FeSOD, CSD1, CSD2, and CCS proteins (Figure 12). When whole plant tissue was examined, *hma5* plants displayed a slight decrease in CSD1, CSD2, and CCS protein expression as well as a small decrease in CSD1 and CSD2 activity in the high copper treatment. MnSOD was used as a loading control and plastocyanin proteins levels

remained relatively unchanged between the mutant and wildtype in both treatments.

4. Discussion

Previously, only a biochemical phenotype based on SOD activity has been described for a *CCS* knockout mutant plant which has been confirmed in this investigation [17]. Visual phenotypes may not have been detected in the past due to the mild defect in growth rate compared to wildtype. However, we have found that when *CCS OX* plants are compared to *CCS* knockout mutants there is a significant difference in growth rate at high copper concentrations. This is most likely due to the increased ability of the plant to compensate for excess copper through sequestration of the ion by the CCS protein. Furthermore, we have detected a chlorophyll fluorescence (Fv/Fm) phenotype of the CCS overexpressing plants. The values of Fv/Fm indicate the level of photoinhibition as a result of damage to photosystem II. High light can cause a dramatic increase in oxidative stress for photosynthetic organisms; however *OX11* and *OX14* plants only displayed a phenotype in treatments with sufficient copper concentrations when CSD proteins are expressed. It has been suggested that SOD enzymes with different metal cofactors function with similar efficiency [38]. Copper, zinc superoxide dismutases have been localized to the cytosol, stroma, and peroxisome, whereas FeSOD has been found in the stroma [1]. Plants may be able to cope with an increase in oxidative stress under sufficient copper concentrations due to the multiple subcellular locations of the three CSD enzymes and at toxic levels the *OX*

plants have the ability to sequester excess metal ions before they become harmful to the cell.

In low copper concentrations, superoxide dismutase expression was slightly affected when CCS expression was altered. Since copper is low in supply the priority targets for the metal are plastocyanin and cytochrome *c* oxidase for photosynthesis and respiration, respectively. As a result, it has been demonstrated that at low copper levels CSD1 and CSD2 have little to no expression and FeSOD is the active enzyme for superoxide dismutase. With the plant below critical toxicity for the metal ion, copper chaperones involved in scavenging and sequestering excess copper are not expressed. At sufficient or toxic levels of copper expression and activity of CSD1 and CSD2 increase and expression and activity of FeSOD are lost. Interestingly, CSD2 expression in *OX* plants is lower than wildtype, whereas CSD1 expression is relatively equal between the same plant lines. The regulatory element for copper within the plant cell has not yet been identified, however if there is an excess of CCS protein scavenging free copper ions this regulatory element in the cytosol and/ or chloroplast would detect a decrease in copper levels. *Arabidopsis* only has one *CCS* gene which encodes two CCS proteins for the cytosol and the stroma [4,17]. It is currently unknown how the plant regulates the transcription of the two versions of the CCS protein. In this investigation, we have overexpressed the full length version of the CCS gene, yet the plant may still be regulating transcription of the two start sites. As a result there may be an increase of the cytosolic version of CCS which is elevating CSD1 levels and decreasing CSD2 expression. Subsequently, the decrease in CSD2

expression may be caused by the degradation of the protein from the lack of cofactor delivery and not a result of a decrease in transcript levels. Since both wildtype and *OX* plants show a slight increase in CCS expression when the copper levels switch from limited to sufficient, this would suggest that the CCS protein is regulated by copper levels in the plant cell.

Since copper requirements vary in different tissue types and at different developmental stages this suggests that changes in expression levels of copper homeostasis proteins over the course of the plant life are expected. Copper requirements, based on protein levels, seem to be the lowest in the root tissue and highest in the inflorescent tissue. Root tissue does not contain chloroplasts which results in the elimination of plastocyanin. With the exclusion of this abundant target for copper there is a decrease in demand for the metal ion. Therefore the plant cell is sufficient in copper and results in the expression of CSD1 and the lack of FeSOD expression in root tissue. However, all photosynthetic tissue contains chloroplasts and consequently more targets for copper within the plant cell.

Plastocyanin is one of the most abundant proteins in photosynthetic tissue and is arguably the most important protein that requires the delivery of copper. With the copper resources going to plastocyanin in vegetative and reproductive tissue the plant cell is easily deficient in copper and FeSOD is the active superoxide dismutase. When copper concentrations in the media are low, CCS protein expression is higher in leaf tissue compared to root tissue before flowering. However, when the plant produces reproductive tissue there is an increase in CCS expression in inflorescent tissue compared to leaf and

root tissues. This may be caused by the requirement of copper for floral development or the increase in oxidative stress generated during reproductive development and plant senescence. It has previously been shown that CCS transcript levels dramatically increase during plant senescence [4]. However, the addition of as little as 2 μM $CuSO_4$ to the media creates a sufficient environment for the plant where tissue specific as well as developmental demands are met. In this treatment, the plants displayed an increase in CCS expression as well as an increase in CSD2 expression in photosynthetic tissue. Even though this level of copper is considered sufficient due to the switch of SOD isoform activity, plastocyanin is still a priority for copper delivery. With much of the copper in the plant cell entering the chloroplast for delivery to plastocyanin this may create a build up of excess copper in the stroma. As a result, chloroplastic CCS and CSD2 expression increases to relieve the stroma from this potentially harmful ion.

Since *OX* plants that display an increase in CCS expression do not have a dramatically altered SOD or lowered expression, what happens to the metal ion that is sequestered by the CCS protein? It is possible that the copper chaperone acts similar to a metallothionein by holding the metal ion until the priority targets in the cell require copper. Alternatively, a more likely scenario may be that CCS has additional delivery targets other than CSD1 and CSD2. Here we have reported a novel interaction between the CCS copper chaperone and the amino terminal domain of HMA5. The HMA5 transporter, similar to RAN1, has two separate ATX-like domains, each with an MxCxxC heavy metal binding domain [14,16]. The multiple copper binding domains

would suggest multiple copper chaperones could deliver ions for translocation by the transporters. As a result both transporters may play an important role in the detoxification of copper from the plant cell. Due to the cytosolic location of CCS as well as the location of HMA5 in the plasma membrane it is likely that an interaction would be seen *in planta*. Compared to the ATX1 and CCHΔ interactions with HMA5, the interaction with CCS was considerably greater when quantitative estimates were measured via β-galactosidase activity assays. However, all three interactions did not appear to be copper dependent. In fact, the interaction with ATX1 seemed to increase as copper availability decreased. Currently, little is known about protein interactions between copper chaperones and transporters involved in metal ion delivery. It is also unknown why some interactions have displayed copper dependency while others have not. This may be due to the protein surfaces involved or the result that fusion tags have on conformation and charge of the proteins that are investigated. However, it is likely that the interaction between the chaperones and transporters occur near the metal binding domain for the ion to transfer from one protein to the other.

With this novel interaction between CCS and HMA5(N), we investigated the effect of a mutation in *HMA5* on CCS, CSD1, and CSD2 expression. Under low copper concentrations there was no difference in protein expression levels between *hma5* mutants and wildtype in root or shoot tissue. Previously, it has been demonstrated that *HMA5* transcript levels are elevated in sufficient copper conditions suggesting that the gene is copper regulated. Additionally, high copper concentrations in root tissue of mutant plants suggest that *hma5*

aids in detoxification of copper in root tissue [14]. As a result, a mutation in *hma5* would not significantly affect copper homeostasis proteins at low copper concentrations. Surprisingly, at high copper concentrations whole plant tissue of *hma5* mutants displayed a decrease in CCS, CSD1, and CSD2 protein expression as well as a decrease in CSD1 and CSD2 activity compared to wildtype. The difference was not great compared to wildtype, but this was an unexpected result. It is possible that HMA5 aids in the long distance transport of copper from root tissue to shoot tissue. If this is the case, it would result in the deficiency of copper in shoot tissue and may lead to a decrease in the transcription of copper regulated genes. If the priority is for delivery to plastocyanin there would be little free copper ions in photosynthetic tissue which could explain a decrease in CCS and CSD expression levels.

With these new findings, we propose that the CCS protein has multiple targets for copper delivery within the plant cell (Figure 13). This copper chaperone can delivery copper to the Cu,ZnSODs as well as HMA5 for cellular detoxification or long distance transport within the organism. It should be noted that a possible visual phenotype was observed in *CCS* knockout mutants growing on soil. This potential phenotype was detected only after flowering occurred in plants that we believe were also limited in copper. After flowering vegetative tissue began to turn yellowish in color and possible defects in floral development could have contributed to a decrease in seed development. Further investigations can be conducted to look at the effects of altering CCS expression on reproductive tissue. Depending on copper concentrations within the cytosol and the stroma the plant will regulate between two different

versions of CCS for different subcellular locations. As cytosolic levels increase CCS can deliver copper to CSD1 and HMA5, whereas when stromal levels increase CCS can deliver copper to CSD2 within the chloroplast.

In this investigation we have reported that the alteration of CCS protein levels slightly affect the expression of the target SOD proteins. We have described a growth phenotype and chlorophyll fluorescence phenotype associated with CCS overexpression. Additionally, that copper concentrations do affect CCS expression slightly which can be seen the best when media switches between limited to sufficient copper. Furthermore, we have identified a novel interaction between the CCS copper chaperone and the HMA5 transporter and propose a new model for copper transport in the plant cell.

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References

[1] Kliebenstein, D.J., Monde, R.A. and Last, R.L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118, 637-50.

[2] Linder, M.C. (1991) Nutritional biochemistry and metabolism : with clinical applications, Elsevier. New York.

[3] Bowler, C., Montagu, M.V. and Inze, D. (1992). Superoxide Dismutase and Stress Tolerance. Annual Review of Plant Physiology and Plant Molecular Biology 43, 83-116.

[4] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., Andres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Lett 579, 2307-12.

[5] Yamasaki, H., Abdel-Ghany, S.E., Cohu, C.M., Kobayashi, Y., Shikanai, T. and Pilon, M. (2007). Regulation of copper homeostasis by microRNA in Arabidopsis. J Biol Chem

[6] Carrington, J.C. and Ambros, V. (2003). Role of microRNAs in plant and animal development. Science 301, 336-8.

[7] Jones-Rhoades, M.W. and Bartel, D.P. (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 14, 787-99.

[8] Sunkar, R. and Zhu, J.K. (2004). Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell 16, 2001-19.

[9] Sunkar, R., Kapoor, A. and Zhu, J.K. (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell 18, 2051-65.

[10] Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D. and Van Montagu, M. (1995). Molecular characterization of a putative Arabidopsis thaliana copper transporter and its yeast homologue. J Biol Chem 270, 28479-86.

[11] Burkhead, J. and Colorado State University. Dept. of Biology. (2003) Copper traffic in plants : roles for newly isolated chloroplast proteins, pp. 145. Colorado State University, Fort Collins, CO.

[12] Puig, S., Mira, H., Dorcey, E., Sancenon, V., Andres-Colas, N., Garcia-Molina, A., Burkhead, J.L., Gogolin, K.A., Abdel-Ghany, S.E., Thiele, D.J., Ecker, J.R., Pilon, M., Penarrubia, L. (2007). Higher plants possess two different types of ATX1-like copper chaperones. Biochem Biophys Res Commun

[13] Himelblau, E., Mira, H., Lin, S.J., Culotta, V.C., Penarrubia, L. and Amasino, R.M. (1998). Identification of a functional homolog of the yeast copper homeostasis gene ATX1 from Arabidopsis. Plant Physiol 117, 1227-34.

[14] Andres-Colas, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker, J.R., Puig, S. and Penarrubia, L. (2006). The

Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J 45, 225-36.

[15] Hirayama, T. and Alonso, J.M. (2000). Ethylene captures a metal! Metal ions are involved in ethylene perception and signal transduction. Plant Cell Physiol 41, 548-55.

[16] Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker J.R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97, 383-93.

[17] Chu, C.C., Lee, W.C., Guo, W.Y., Pan, S.M., Chen, L.J., Li, H.M. and Jinn, T.L. (2005). A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. Plant Physiol 139, 425-36.

[18] Lamb, A.L., Wernimont, A.K., Pufahl, R.A., O'Halloran, T.V. and Rosenzweig, A.C. (2000). Crystal structure of the second domain of the human copper chaperone for superoxide dismutase. Biochemistry 39, 1589-95.

[19] Lamb, A.L., Wernimont, A.K., Pufahl, R.A., Culotta, V.C., O'Halloran, T.V. and Rosenzweig, A.C. (1999). Crystal structure of the copper chaperone for superoxide dismutase. Nat Struct Biol 6, 724-9.

[20] Hall, L.T., Sanchez, R.J., Holloway, S.P., Zhu, H., Stine, J.E., Lyons, T.J., Demeler, B., Schirf, V.., Hansen, J.C., Nersissian, A.M., Valentine, J.S., and Hart, P.J. (2000). X-ray crystallographic and analytical ultracentrifugation analyses of truncated and full-length yeast copper chaperones for SOD (LYS7): a dimer-dimer model of LYS7-SOD association and copper delivery. Biochemistry 39, 3611-23.

[21] Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

[22] Clough, S.J. and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16, 735-43.

[23] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., Andres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Letters 579, 2307-2312.

[24] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-54.

[25] Beauchamp, C. and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44, 276-87.

[26] Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M. and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17, 1233-51.

[27] James, P., Halladay, J. and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425-36.
[28] Clontech. (2001) Yeast Protocols Handbook. Mountain View, CA.
[29] Miller, J.H. (1972) Experiments in Molecular Geneticsed.^eds). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[30] Miller, J.H. (1992). In A Short Course in Bacterial Genetics ed.^eds), pp. 74. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[31] Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G. and Thompson, J.D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31, 3497-500.
[32] Peitsch, M.C. (1995). Protein modeling by E-mail. Bio/Technology 13, 658-660.
[33] Guex, N. and Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714-23.
[34] Schwede, T., Kopp, J., Guex, N. and Peitsch, M.C. (2003). SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 31, 3381-5.
[35] Kopp, J. and Schwede, T. (2004). The SWISS-MODEL Repository of annotated three-dimensional protein structure homology models. Nucleic Acids Res 32, D230-4.
[36] Burkhead, J.L., Abdel-Ghany, S.E., Morrill, J.M., Pilon-Smits, E.A. and Pilon, M. (2003). The Arabidopsis thaliana CUTA gene encodes an evolutionarily conserved copper binding chloroplast protein. Plant J 34, 856-67.
[37] Day, I.S., Miller, C., Golovkin, M. and Reddy, A.S. (2000). Interaction of a kinesin-like calmodulin-binding protein with a protein kinase. J Biol Chem 275, 13737-45.
[38] Fridovich, I. (1978). The biology of oxygen radicals. Science 201, 875-80.

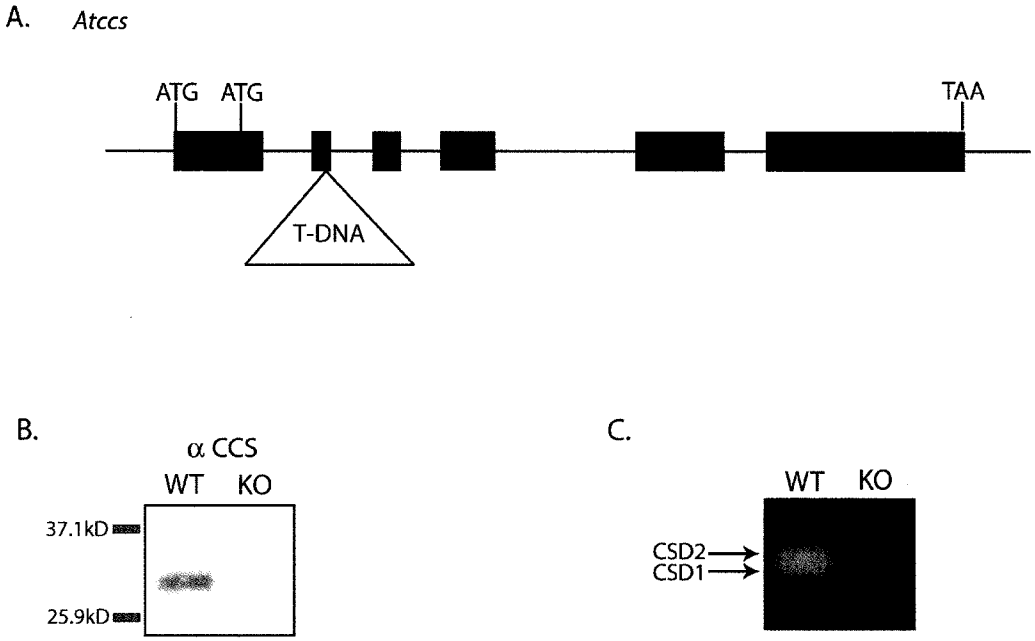

Figure 1. *CCS* knockout plants loose expression of CCS and activity of CSD1 and CSD2. A map illustrating the *AtCCS* gene and the location of the T-DNA insert in the *CCS* knockout mutant plant (Panel A). Wildtype and knockout plants were grown on soil for 6-8 weeks and soluble protein was extracted for immunoblot and SOD activity analysis. A total of 10 µg of soluble protein from whole plant tissue was resolved on 12.5% SDS-PAGE gels for CCS expression (Panel B). SOD activity was analyzed using the same tissue (Panel C). A total of 10 µg of soluble protein was resolved on a 10-20% Tris-HCL gradient gel for analysis of CSD1 and CSD2 activity.

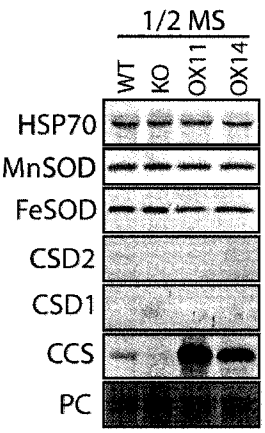

Figure 2. *CCS* overexpressing plants accumulate high levels of CCS protein. Wildtype (WT), *KO*, *OX11*, and *OX14* plants were grown for 14 days on ½ MS media for immunoblot analysis. A total of 10 µg of soluble protein from whole plant tissue was resolved on 12.5% SDS-PAGE gels for protein expression. MnSOD protein was used as a loading control.

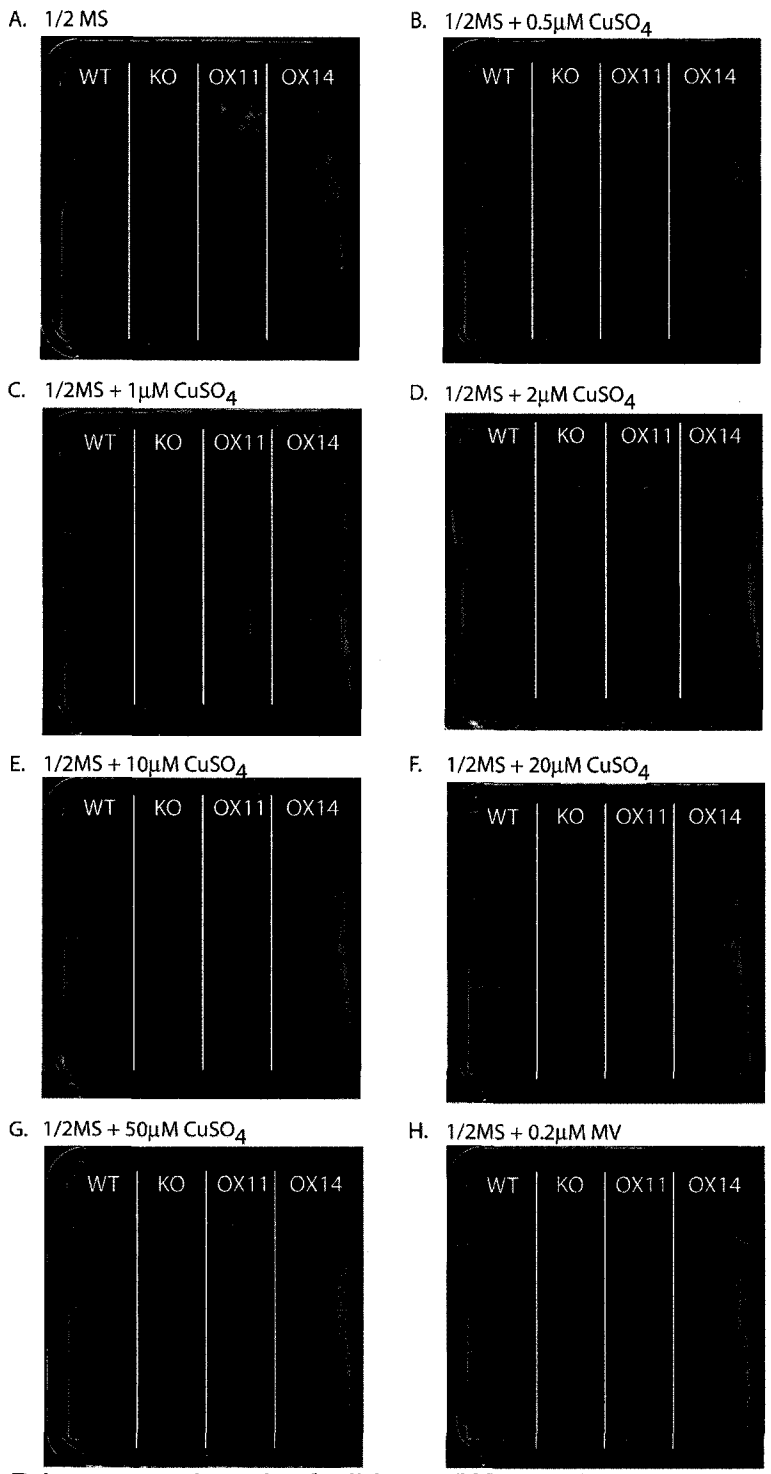

Figure 3. Primary root length of wildtype (WT), *CCS* knockout, *OX11*, and *OX14* plants grown in ½ MS for 10 days in ½ MS or ½ MS media supplemented with $CuSO_4$ or methyl viologen (MV) where indicated.

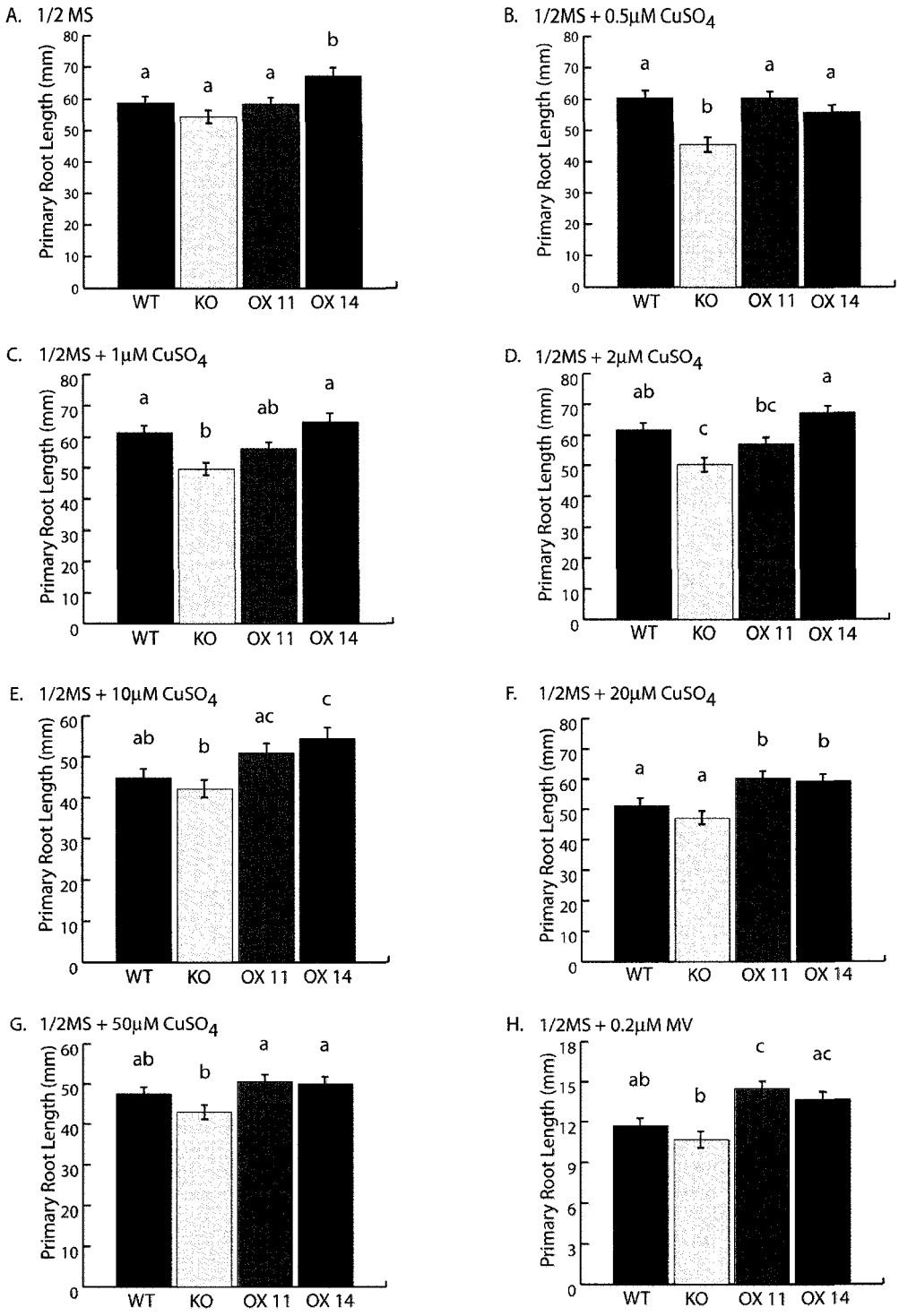

Figure 4. Graphs of mean primary root length ± S.E. of wildtype, *KO*, *OX11*, and *OX14* plants grown for 10 days in ½ MS or ½ MS media supplemented with $CuSO_4$ or methyl viologen (MV). Letters denote significant difference based on Tukey's HSD comparison of least squared means (ANOVA) (n = 17-29 individual measurements, p = 0.05).

	Before High Light Treatment				After High Light Treatment			
	Fv/Fm	ΦPSII	qP	NPQ	Fv/Fm	ΦPSII	qP	NPQ
1/2MS								
WT	0.89 ± 0.01	0.45 ± 0.01^{a}	0.64 ± 0.01^{a}	0.80 ± 0.03^{ab}	0.81 ± 0.02	0.46 ± 0.01	0.69 ± 0.01^{a}	0.52 ± 0.05
KO	0.90 ± 0.01	0.46 ± 0.01^{ab}	0.66 ± 0.01^{a}	0.83 ± 0.03^{a}	0.83 ± 0.02	0.49 ± 0.02	0.73 ± 0.01^{b}	0.58 ± 0.06
OX11	0.90 ± 0.01	0.50 ± 0.01^{b}	0.69 ± 0.01^{b}	0.71 ± 0.03^{b}	0.77 ± 0.02	0.44 ± 0.02	0.70 ± 0.01^{ab}	0.47 ± 0.06
OX14	0.90 ± 0.01	0.47 ± 0.01^{ab}	0.66 ± 0.01^{a}	0.72 ± 0.03^{ab}	0.81 ± 0.02	0.47 ± 0.02	0.71 ± 0.01^{ab}	0.53 ± 0.06
1/2MS + 0.5 μM $CuSO_4$								
WT	0.89 ± 0.01^{a}	0.47 ± 0.01	0.66 ± 0.01	0.72 ± 0.03^{a}	0.89 ± 0.01	0.54 ± 0.01^{a}	0.74 ± 0.01^{a}	1.00 ± 0.03
KO	0.89 ± 0.01^{a}	0.46 ± 0.01	0.65 ± 0.01	0.89 ± 0.03^{b}	0.90 ± 0.01	0.56 ± 0.01^{ab}	0.75 ± 0.01^{b}	1.10 ± 0.03
OX11	0.90 ± 0.01^{b}	0.50 ± 0.01	0.68 ± 0.01	0.79 ± 0.03^{a}	0.90 ± 0.01	0.58 ± 0.01^{b}	0.77 ± 0.01^{a}	0.98 ± 0.04
OX14	0.89 ± 0.01^{a}	0.47 ± 0.01	0.66 ± 0.01	0.82 ± 0.03^{ab}	0.88 ± 0.01	0.53 ± 0.01^{a}	0.74 ± 0.01^{a}	1.01 ± 0.03
1/2MS + 1 μM $CuSO_4$								
WT	0.89 ± 0.01^{a}	0.45 ± 0.01^{a}	0.64 ± 0.01^{a}	0.75 ± 0.03^{a}	0.77 ± 0.01	0.41 ± 0.02	0.68 ± 0.02	0.61 ± 0.04
KO	0.89 ± 0.01^{a}	0.48 ± 0.01^{ab}	0.67 ± 0.01^{bc}	0.90 ± 0.03^{b}	0.77 ± 0.02	0.40 ± 0.02	0.66 ± 0.02	0.51 ± 0.05
OX11	0.90 ± 0.01^{b}	0.50 ± 0.01^{b}	0.69 ± 0.01^{c}	0.75 ± 0.03^{a}	0.78 ± 0.01	0.44 ± 0.02	0.70 ± 0.02	0.60 ± 0.04
OX14	0.89 ± 0.01^{ab}	0.45 ± 0.01^{a}	0.65 ± 0.01^{ab}	0.81 ± 0.03^{ab}	0.81 ± 0.01	0.42 ± 0.01	0.67 ± 0.01	0.61 ± 0.04
1/2MS + 2 μM $CuSO_4$								
WT	0.90 ± 0.01^{a}	0.46 ± 0.01^{a}	0.65 ± 0.01^{a}	0.77 ± 0.03	0.84 ± 0.01^{ab}	0.49 ± 0.02^{a}	0.74 ± 0.02^{a}	0.88 ± 0.06^{a}
KO	0.89 ± 0.01^{a}	0.49 ± 0.01^{ab}	0.68 ± 0.01^{b}	0.86 ± 0.03	0.81 ± 0.01^{b}	0.50 ± 0.02^{ab}	0.78 ± 0.01^{ab}	1.12 ± 0.06^{b}
OX11	0.90 ± 0.01^{b}	0.52 ± 0.01^{b}	0.71 ± 0.01^{b}	0.76 ± 0.04	0.87 ± 0.01^{a}	0.57 ± 0.02^{b}	0.81 ± 0.02^{b}	1.08 ± 0.06^{ab}
OX14	0.90 ± 0.01^{ab}	0.46 ± 0.01^{a}	0.65 ± 0.01^{a}	0.82 ± 0.03	0.85 ± 0.01^{ab}	0.52 ± 0.02^{ab}	0.76 ± 0.01^{ab}	1.05 ± 0.06^{ab}
1/2MS + 10 μM $CuSO_4$								
WT	0.89 ± 0.01^{a}	0.47 ± 0.01^{a}	0.65 ± 0.01^{a}	0.72 ± 0.03^{a}	0.82 ± 0.02^{ab}	0.49 ± 0.02^{ab}	0.73 ± 0.01^{a}	0.90 ± 0.07
KO	0.89 ± 0.01^{a}	0.47 ± 0.01^{a}	0.66 ± 0.01^{a}	0.89 ± 0.03^{b}	0.77 ± 0.02^{b}	0.44 ± 0.02^{b}	0.71 ± 0.01^{a}	0.70 ± 0.08
OX11	0.90 ± 0.01^{b}	0.53 ± 0.01^{b}	0.71 ± 0.01^{b}	0.71 ± 0.03^{a}	0.83 ± 0.02^{a}	0.55 ± 0.02^{a}	0.79 ± 0.01^{b}	0.83 ± 0.06
OX14	0.89 ± 0.01^{a}	0.49 ± 0.01^{ab}	0.68 ± 0.01^{ab}	0.78 ± 0.03^{ab}	0.84 ± 0.02^{ab}	0.51 ± 0.02^{ab}	0.73 ± 0.01^{a}	0.81 ± 0.06
1/2MS + 20 μM $CuSO_4$								
WT	0.89 ± 0.01^{a}	0.49 ± 0.01	0.67 ± 0.01^{a}	0.81 ± 0.03	0.79 ± 0.03^{ab}	0.48 ± 0.02^{a}	0.74 ± 0.02	0.67 ± 0.08^{a}
KO	0.89 ± 0.01^{a}	0.53 ± 0.01	0.72 ± 0.01^{b}	0.82 ± 0.03	0.76 ± 0.02^{b}	0.49 ± 0.02^{a}	0.77 ± 0.01	0.70 ± 0.07^{a}
OX11	0.90 ± 0.01^{b}	0.50 ± 0.01	0.68 ± 0.01^{ab}	0.77 ± 0.03	0.87 ± 0.02^{a}	0.57 ± 0.02^{b}	0.79 ± 0.01	1.00 ± 0.06^{b}
OX14	0.89 ± 0.001^{ab}	0.49 ± 0.01	0.67 ± 0.01^{a}	0.76 ± 0.03	0.83 ± 0.02^{a}	0.53 ± 0.02^{ab}	0.77 ± 0.01	0.90 ± 0.06^{ab}
1/2MS + 50 μM $CuSO_4$								
WT	0.89 ± 0.01^{a}	0.48 ± 0.02^{ab}	0.65 ± 0.02^{ab}	0.88 ± 0.05	0.76 ± 0.03	0.45 ± 0.03	0.75 ± 0.02^{ab}	0.80 ± 0.11
KO	0.89 ± 0.01^{a}	0.50 ± 0.02^{ab}	0.68 ± 0.02^{ab}	1.01 ± 0.05	0.76 ± 0.02	0.48 ± 0.02	0.78 ± 0.02^{a}	0.82 ± 0.09
OX11	0.90 ± 0.01^{b}	0.53 ± 0.02^{b}	0.70 ± 0.01^{a}	0.92 ± 0.04	0.78 ± 0.03	0.47 ± 0.02	0.73 ± 0.02^{ab}	0.64 ± 0.10
OX14	0.89 ± 0.01^{a}	0.47 ± 0.01^{a}	0.64 ± 0.01^{a}	0.89 ± 0.04	0.77 ± 0.03	0.43 ± 0.03	0.69 ± 0.02^{b}	0.58 ± 0.11
1/2MS + 0.2 μM MV								
WT	0.88 ± 0.01	0.47 ± 0.01	0.66 ± 0.01	0.83 ± 0.08^{a}	0.81 ± 0.02	0.42 ± 0.02	0.65 ± 0.02	1.02 ± 0.08
KO	0.89 ± 0.01	0.47 ± 0.02	0.67 ± 0.02	1.47 ± 0.09^{b}	0.84 ± 0.02	0.45 ± 0.02	0.70 ± 0.03	1.10 ± 0.09
OX11	0.88 ± 0.01	0.48 ± 0.01	0.67 ± 0.01	1.03 ± 0.07^{a}	0.86 ± 0.02	0.45 ± 0.02	0.68 ± 0.02	1.22 ± 0.07
OX14	0.87 ± 0.01	0.47 ± 0.02	0.66 ± 0.02	0.88 ± 0.09^{a}	0.82 ± 0.02	0.43 ± 0.02	0.68 ± 0.02	1.14 ± 0.07

Table 1. Measurements of mean chlorophyll fluorescence (Fv/Fm, ΦPSII, qP, and NPQ) before and after high light treatments ± S.E. Letters denote significant difference of plants within treatments based on Tukey's HSD comparison of least squared means (ANOVA) (n = 3 - 5 individual measurements, p = 0.10). Absences of letters indicate no significant difference between plants within the treatment.

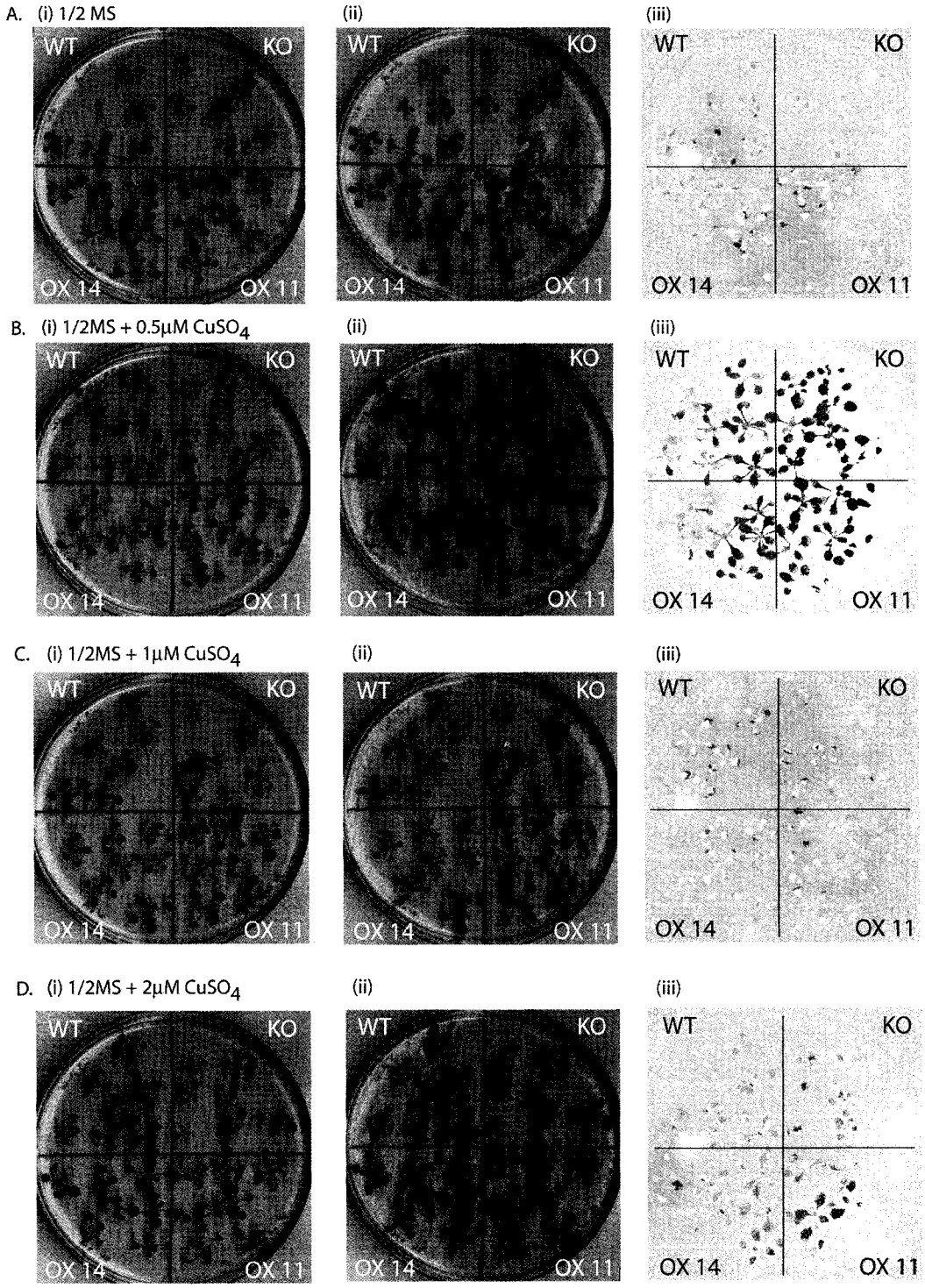

Figure 5 (Panels A-D). Images of wildtype, *KO*, *OX11*, and *OX14* plants grown in ½ MS and ½ MS media supplemented with $CuSO_4$ or MV. Plants were grown for 14 days (i), treated to 64 hours of high light (ii), and measured for chlorophyll fluorescence (iii). Chlorophyll fluorescence images represent Fv/Fm values and are falsely colored (Blue, min value = 0.45; Red, max value = 0.95).

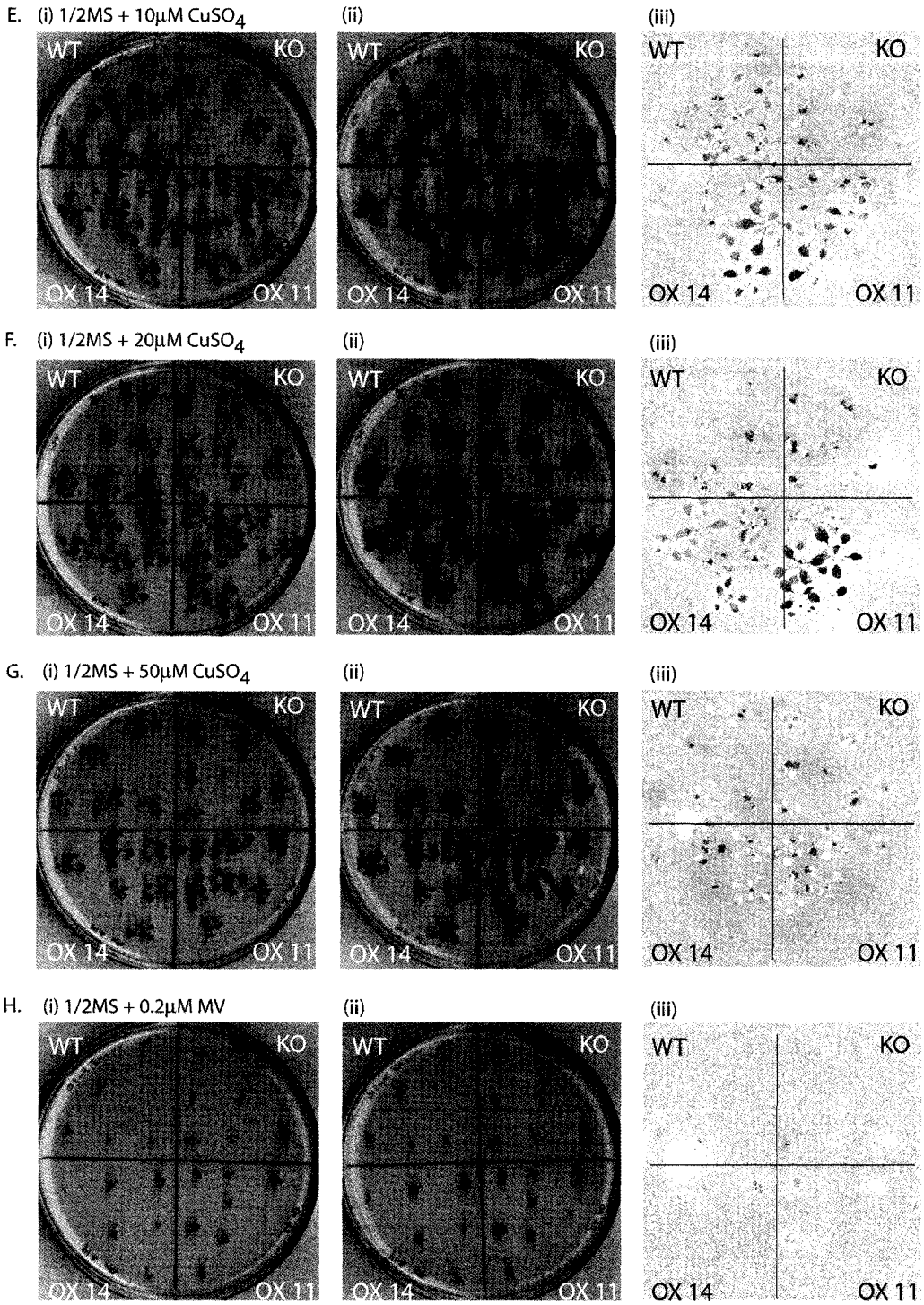

Figure 5 (Panels E-H). Images of wildtype, *KO*, *OX11*, and *OX14* plants grown in ½ MS and ½ MS media supplemented with $CuSO_4$ or MV. Plants were grown for 14 days (i), treated to 64 hours of high light (ii), and measured for chlorophyll fluorescence (iii). Chlorophyll fluorescence images represent Fv/Fm values and are falsely colored (Blue, min value = 0.45; Red, max value = 0.95).

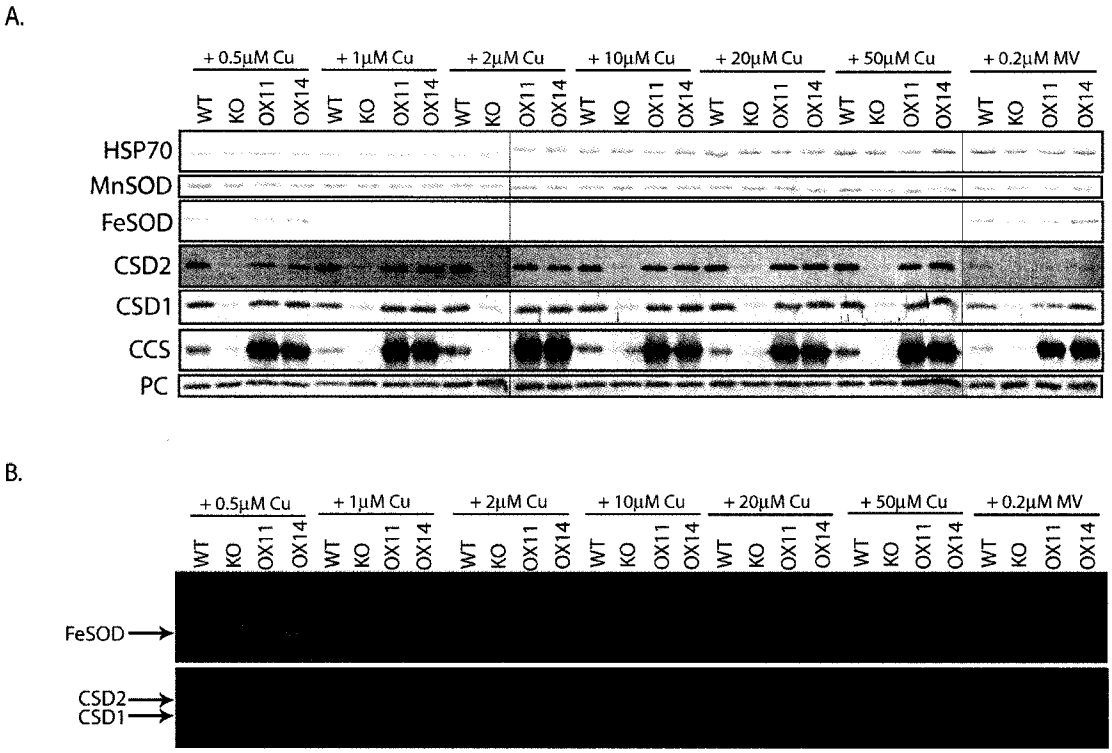

Figure 6. Altered CCS expression does not dramatically affect copper homeostasis protein expression or SOD activity. Immunoblot and SOD activity analysis of wildtype (WT), *CCS* knockout (KO), *OX11* and *OX14* plants grown for 10 days in ½ MS supplemented with 0.5 - 50 μM $CuSO_4$ or 0.2 μM MV. A total of 10 μg of soluble protein from whole plant tissue was resolved on 12.5% SDS-PAGE gels for protein expression (Panel A). MnSOD and HSP70 proteins were used as loading controls. SOD activity was analyzed using the same tissue (Panel B). A total of 10 μg of soluble protein was resolved on a 15% NATIVE-PAGE gel for FeSOD analysis and on a 10-20% Tris-HCL gradient gel for analysis of CSD1 and CSD2 activity.

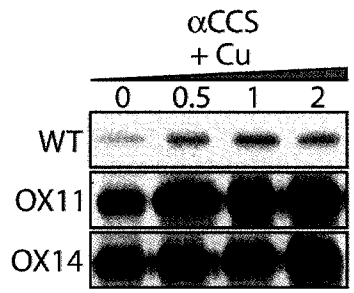

Figure 7. Copper concentrations in the media affect CCS protein expression levels. Immunoblot analysis of wildtype (WT), *OX11*, and *OX14* plant lines grown for 10 days in ½ MS and ½ MS supplemented with 0.5 -2 μM $CuSO_4$.

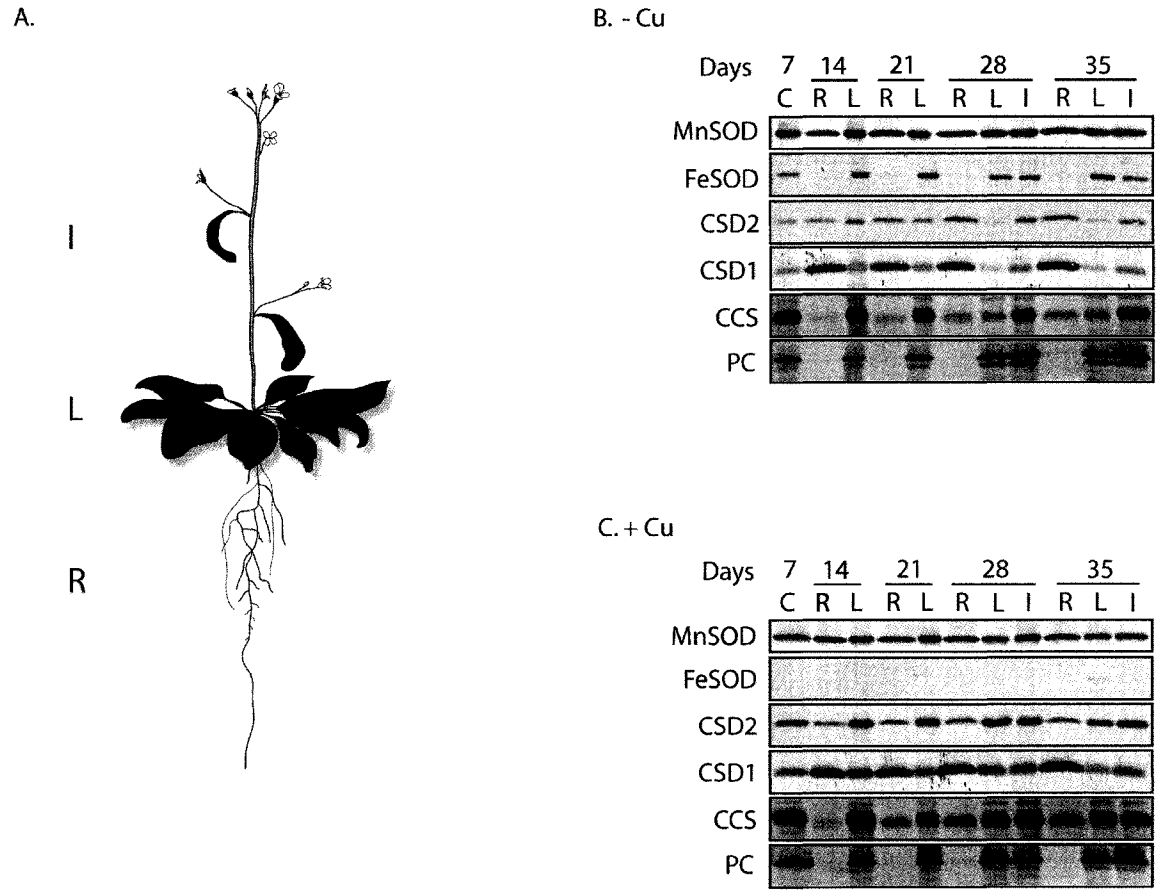

Figure 8. CCS expression varies in different tissue type and at different developmental stages. An illustration of *Arabidopsis* depicting tissue that was harvested (R, roots; L, leaves; I, inflorescence) (Panel A). Immunoblot analysis of wildtype plants grown for 35 days in ½ MS (Panel B) and ½ MS supplemented with 2 µM $CuSO_4$ (Panel C) (C, cotyledon). Tissue was dissected and harvested separately for analysis and a total of 10 µg of soluble protein from root, leaf, or inflorescent tissue was resolved on 12.5% SDS-PAGE gels for protein expression.

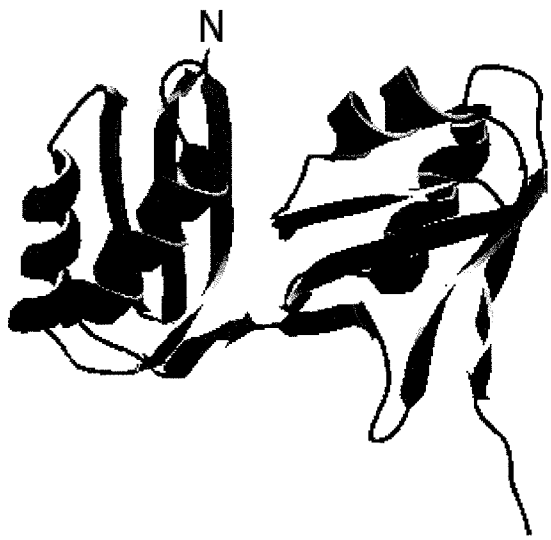

Figure 9. A model of the amino terminal domain of HMA5(N) depicting two separate ATX-like structural domains. This image was produced using the SWISS-MODEL Server and SwissPDB-viewer software.

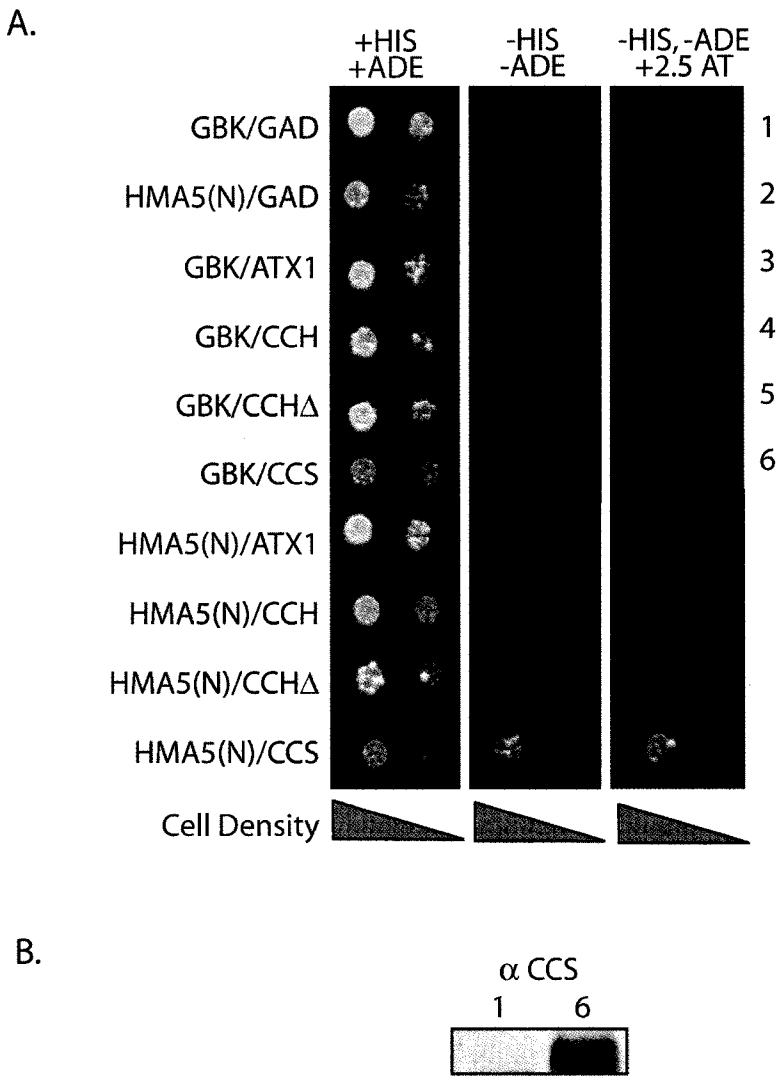

Figure 10. Interaction between the HMA5(N) transporter and copper chaperones using yeast two-hybrid. A yeast two-hybrid spot assay was conducted using the amino terminal domain of HMA5 and ATX1, CCH, and CCS copper chaperones. AH109 yeast cells were co-transformed with pGAD- and pGBK- based plasmids containing encoding regions of proteins. Cells were grown in liquid cultures and spotted on plates containing +HIS+ADE, -HIS-ADE, and -HIS-ADE supplemented with 2.5 mM 3-amino-1,2,4-triazole (AT) (Panel A). CCS fusion protein is expressed in yeast cells (Panel B). Yeast transformants were analyzed by immunoblot analysis with CCS antibody. Size of protein corresponds to the appropriate molecular weight of purified full length CCS protein fused to a yeast two-hybrid tag.

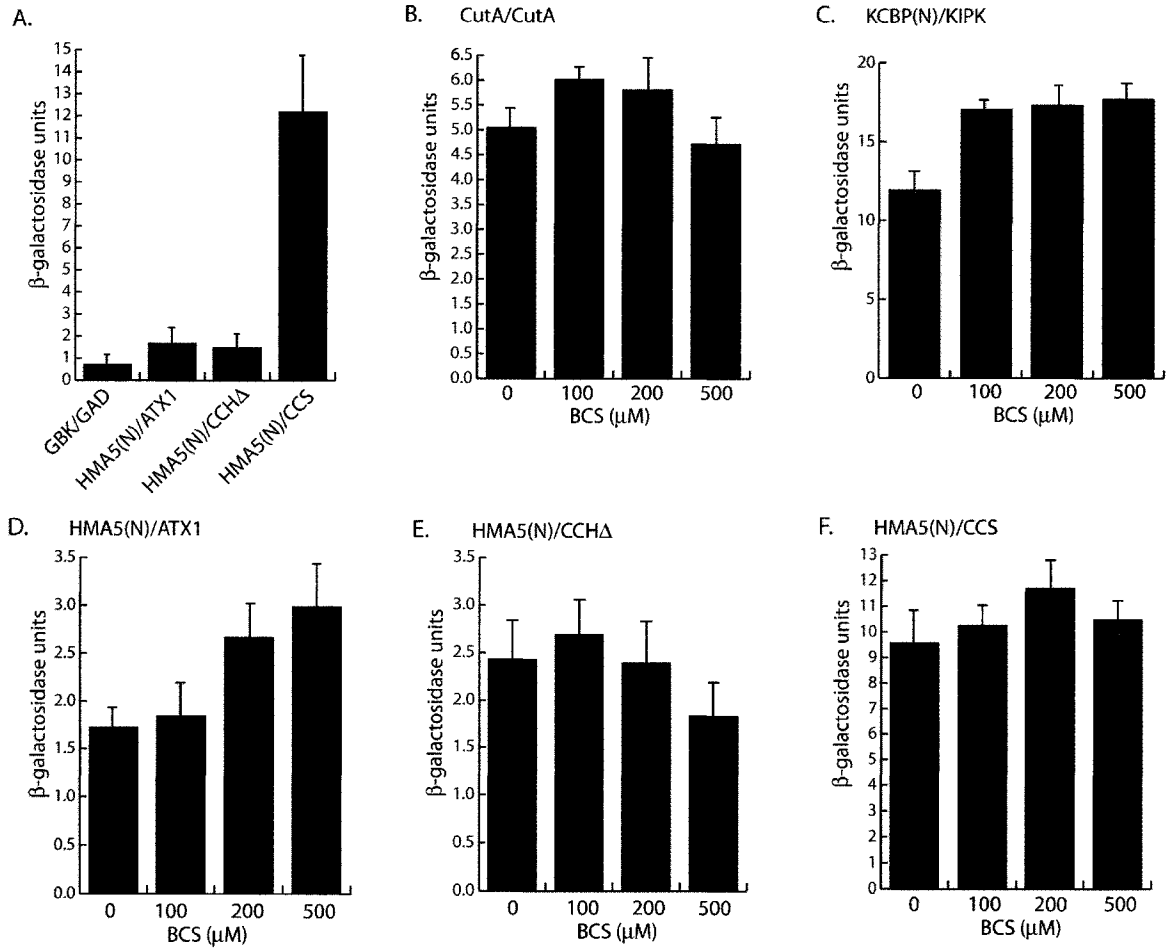

Figure 11. Quantitative estimates of the interactions between HMA5(N) and copper chaperones showing no copper dependence. AH109 yeast cells were co-transformed and grown in liquid cultures for β-galactosidase activity measurements (Panel A). Bathocuproine disulfonic acid (BCS) was added to the media to test copper dependency (Panels B-F). The CutA/CutA and KCBP(N)/KIPK interactions were used as a copper independent control.

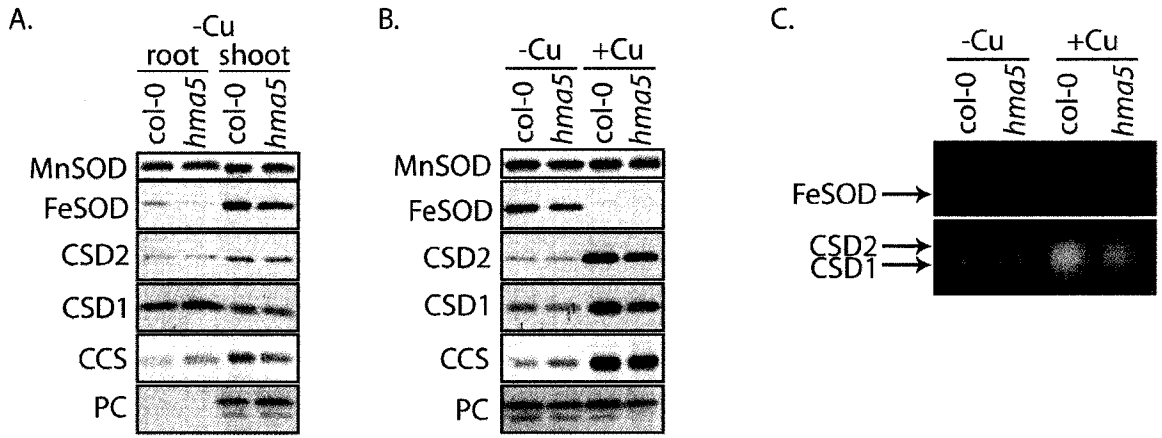

Figure 12. SOD and CCS expression is slightly affected by a mutation in *HMA5*. Immunoblot analysis of wildtype (WT), and *hma5* plants grown for 14 days in ½ MS and ½ MS supplemented with 10 µM $CuSO_4$. Tissue was harvested from ½ MS media and dissected for analysis. A total of 10 µg of soluble protein from root or shoot tissue was resolved on 12.5% SDS-PAGE gels for protein expression (Panel A). Whole tissue was harvested from low and high copper treatments and 10 µg of soluble protein was resolved on 12.5% SDS-PAGE gels for protein expression (Panel B). MnSOD protein was used as a loading control. SOD activity was analyzed using the same whole plant tissue (Panel C). A total of 10 µg of soluble protein was resolved on a 15% NATIVE-PAGE gel for FeSOD analysis and on a 10-20% Tris-HCL gradient gel for analysis of CSD1 and CSD2 activity.

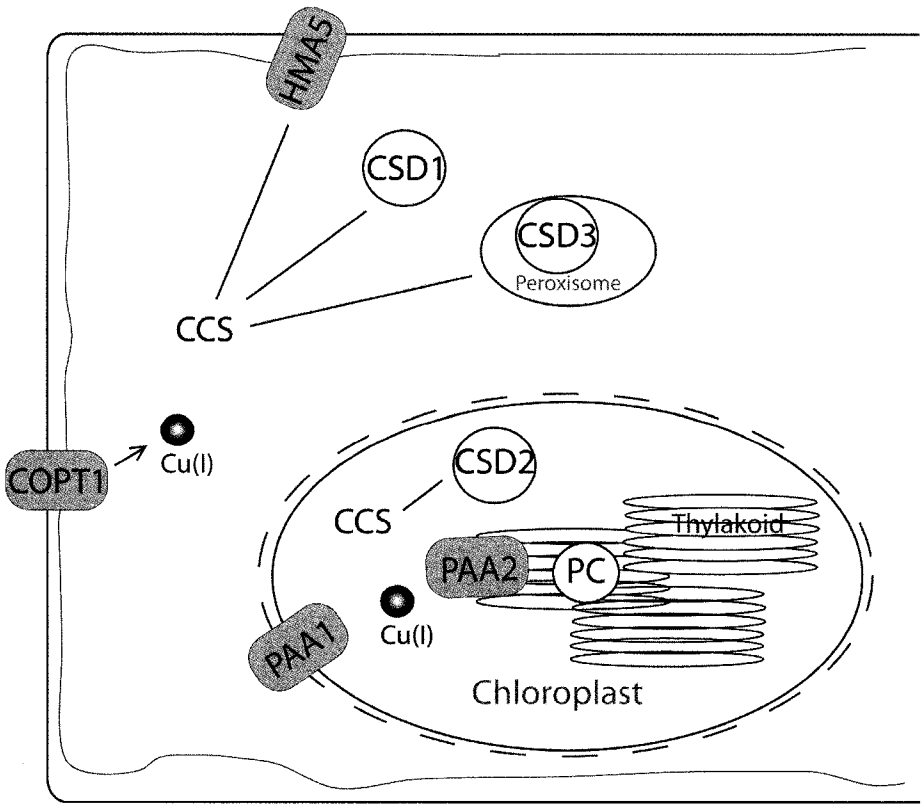

Figure 13. A new model for copper transport in the plant cell based on a novel interaction between the CCS copper chaperone and the HMA5 transporter.

CHAPTER 5

HMA1 functions to transport a metal other than Cu(I) across the chloroplast envelope which affects photosynthetic activity

Abstract

P_{1B}-type ATPase transporters, a total of eight in *Arabidopsis*, are responsible for the translocation of metal ions across membranes. Several of these proteins are involved in copper transport and homeostasis in plants. Copper is an essential micronutrient that is required for both photosynthesis and respiration; transport of metal ions, especially copper, must be tightly regulated due to potential toxic effects. Two P_{1B}-type ATPases, PAA1 and PAA2, have been described and are functional homologs of the CtaA and PacS in cyanobacteria. They have been localized to the chloroplast envelope and thylakoid membrane, respectively, and are importers of copper into the organelle for CSD2 and PC function. Recently, the HMA1 transporter has been localized to the chloroplast membrane and some data suggests that it could transport copper for CSD2 activity. Here we compare *hma1* mutants to *paa1* and *paa2* for chlorophyll fluorescence, protein expression, and chloroplast structure. We report that all mutants contain a chlorophyll fluorescence

phenotype which affects photosystem II efficiency. However, this phenotype can be recovered by copper feeding in *paa1* and *paa2* plants, but not *hma1* mutants. Furthermore, protein levels of CSD1, CSD2, CCS, and PC were not affected in *hma1* mutants and there was no difference in SOD activity between *hma1* and control plants. Additionally, we describe a unique change in chloroplast structure of *hma1-4* mutants that is distinctly different from wildtype and *paa1-3* plants. With this data, we propose that HMA1 transports a metal ion across the chloroplast envelope other than Cu(I) which affects photosynthetic activity.

Abbreviations used:

CCS, copper chaperone for Cu/Zn superoxide dismutase; Cu,ZnSOD, Cu,Zn superoxide dismutase; CSD1, cytosolic Cu,Zn SOD; CSD2, stromal Cu,Zn SOD; FeSOD, Fe superoxide dismutase; F_v/F_m, the quantum efficiency of open photosystem II centers; MnSOD, Mn superoxide dismutase; NPQ, Non-photochemical quenching; PC, plastocyanin; PSII, Photosystem II; ΦPSII, Quantum yield of photosystem II photochemistry; qP, Photochemical quenching; SOD, superoxide dismutase.

1. Introduction

The Heavy Metal Associated gene family encodes proteins that are categorized as P_{1B}-type ATPases. These transporters, a total of eight in *Arabidopsis*, are responsible for the translocation of metal ions across membranes. P-type ATPase proteins are predicted to have eight

transmembrane domains with a large cytosolic loop between the sixth and seventh transmembrane domain [1,2]. There is a heavy metal binding domain at the amino and/or carboxy termini as well as a CPx motif in the sixth transmembrane domain that is speculated to function in ion transduction [3,4]. Another conserved sequence is DKTGT, found in the large cytosolic loop, which is the site of phosphorylation. Once a metal binds to the transporter, ATP is required for phosphorylation that changes the conformation of the protein resulting in the transfer of an ion across the membrane [5].

The P_{1B}-type ATPase subgroup of transporters is further divided into two different groups based on the putative heavy metals they are hypothesized to transport. The first group is the $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ ion transporters and consists of HMA1, HMA2, HMA3, and HMA4 in *Arabidopsis*; while the second group includes HMA5, PAA1 (HMA6), RAN1 (HMA7), and PAA2 (HMA8) and is thought to transport Cu^{2+}/Ag^{2+} ions [6,7]. These two groups were originally developed based on sequence and phylogenetic analysis with the transporters in the Zn group containing histidine rich regions in the amino and/or carboxy termini areas of the proteins, whereas the transporters in the Cu group have one or two MxCxxC heavy metal binding domains in their amino terminus region.

Copper is an essential micronutrient that is required for the biological processes of photosynthesis and respiration. However, homeostasis of this metal ion is tightly regulated in organisms due to its potential toxic effects. Copper is thought to enter the plant cell membrane from the apoplast by a Ctr-like transporter called COPT1 [8]. Once in the cytosol, copper is then

transported to one of its many targets via copper chaperone. *Arabidopsis* contains three Cu,Zn SOD enzymes located in the cytosol (CSD1), stroma (CSD2), and peroxisome (CSD3). Plants possess a homolog of the yeast CCS, also called CCS that is the copper chaperone for SOD.

The copper chaperones responsible for delivery to the mitochondria for cytochrome *c* oxidase and the chloroplast envelope are currently unknown; however several chloroplastic P_{1B}-type ATPases have been described. PAA1 has been localized to the chloroplast envelope and is thought to be the primary transporter for copper ions into the organelle [9]. The PAA2 transporter, a homolog of PAA1, has been localized to the thylakoid membrane and is responsible for copper transport into the lumen where it is required for the proper function of plastocyanin [10]. The *PAA1* gene is expressed in the root tissue, whereas *PAA1* and *PAA2* are both expressed in photosynthetic tissue. Plants containing mutations in the *PAA1* or *PAA2* genes display a decrease in growth rate and exhibit a high chlorophyll fluorescence phenotype that suggests a defect in the electron transport pathway. Both mutants also show a decreased protein accumulation of plastocyanin which may be correlated to the growth rate and fluorescence phenotypes. Interestingly, these phenotypes can be overcome by copper feeding [9,10].

Since a mutation in *PAA1* is not lethal and only results in a decrease in growth rate due to impairment of photosynthetic activity it has been hypothesized that copper can enter the chloroplast through non-specific interaction with another transporter. Recently, a third P_{1B}-type ATPase, HMA1, has been localized to the chloroplast [11]. Yeast expression data has

suggested that HMA1 could be involved in both copper and zinc transport across the envelope. In *Arabidopsis*, a mutation in *HMA1* causes leaf chlorosis in high light treatments a reported decrease in SOD activity [11]. It has been proposed that HMA1 could be an alternate copper transporter in the chloroplast envelope that translocates the ion for CSD2 activity and function.

Comparison of HMA1 and PAA transporter sequences yield several dissimilarities. The PAA transporters have a typical heavy metal binding motif in the amino terminal domain that is associated with copper transport, MxCxxC (Figure 1). Other copper transporters, RAN1 and HMA5, as well as copper chaperones also contain MxCxxC or CxxC binding domains. In contrast, the HMA1 transporter has a histidine rich region at the amino terminus. Additionally, the PAA transporters contain a CPC sequence in the sixth transmembrane domain, whereas HMA1 contains a CPS motif in the site of ion transduction. Although, the HMA1 protein sequence is dissimilar to PAA1 and PAA2 it is not highly similar to other putative zinc transporters either. The sequence of HMA1 is slightly different from HMA2, 3, and 4 in that the hisitidine rich region is in the amino terminus and it has a rather short carboxy terminus. Since the hypothesis of the $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ and Cu^{2+}/Ag^{2+} groups of Heavy Metal Associated transporters there has been a significant amount of experimental data that supports the theory. The more recent data to suggest that HMA1 could be a copper transporter rather than a Zn transporter [11] has created much dispute over the function of this protein.

In this investigation, we compare the *hma1* mutants to *paa1* and *paa2* mutants. If all three transporters reside in the chloroplast and transport copper

we hypothesize that mutations in those genes would create similar physiological and biochemical effects on the plants. In particular, other proteins involved in copper homeostasis would be affected to compensate for the mutation. We report that a mutation in *HMA1* creates a chlorophyll fluorescence phenotype that is unlike *paa1* and *paa2* in that it is not affected by copper feeding. Additionally, we demonstrate that the proteins involved in copper homeostasis are not affected in *hma1* mutants in expression levels or activity. Finally, we describe a unique change in chloroplast structure in *hma1* plants that is different than *paa1* mutants. All together, this data suggests that the HMA1 transporter functions in the translocation of an ion other than Cu(I) across the chloroplast envelope and that affects photosynthetic activity.

2. Methods and Materials

Plant material and growth conditions. Arabidopsis ecotypes Colombia-O (Col) and Wassilewskija (Ws) were used as wildtype controls. The *hma1* mutant seed, *hma1-4* (Col), ACT (Ws), and DRC (Ws), were obtained from Norbert Rolland (Université Joseph Fourier, Grenoble, France), were propagated for this study, and insertions were confirmed using polymerase chain reaction (PCR). The pc-2 (pete2) seed were obtained from Dario Leister (Max-Planck-Institut für Züchtungsforschung, Köln, Germany) and has been described [12]. The *paa1-3*, and *paa2-1* mutants have been described [9,10]. *Arabidopsis* were either grown in soil or one half Murashige and Skoog (MS) medium including 1% sucrose and 0.4% agar gel [13]. The agar medium was supplemented with $CuSO_4$ (Sigma # C9012; Sigma, St Louis) as indicated.

Chlorophyll Fluorescence Measurements. Chlorophyll fluorescence measurements were taken for plants grown in normal light conditions, high light, as well as copper feeding treatments. Seeds were sown in soil for normal light conditions grown for four weeks at a light intensity of 120 µmol photons $m^{-2}\cdot s^{-1}$ and 25°C. Plants in the high light experiment were also grown on soil for a total of four weeks, two weeks at a light intensity of 120 µmol photons $m^{-2}\cdot s^{-1}$ and two weeks at a light intensity of 250 µmol photons $m^{-2}\cdot s^{-1}$ and 25°C. For the copper feeding experiment, plants were grown in one half MS medium with 1% agarose for 14 days in 12/12hr light/dark cycles at a light intensity of 120 µmol photons $m^{-2}\cdot s^{-1}$ and 23°C. Data from plants on one half MS media was taken from two separate biological replicates and was compiled. Chlorophyll fluorescence was imaged using a Fluorcam 700MF, controlled by v. 5.0 PSI Fluorcam software (Photon System Instruments, Brno, Czech Republic), on one hour dark-adapted plants. Statistical analysis was performed using the Jump-in software package (SAS Institute, Cory, NC).

Protein Analysis by Immunoblotting and Native Assays. Soluble protein extraction of plant tissue for SDS-PAGE and NATIVE-PAGE analysis was performed according to [14]. Protein was quantified using the Bradford method [15] and bovine serum albumin was used for a standard curve. A total of 15 µg of protein extract was fractionated on 12.5% SDS-PAGE gels before immunodetection. While 10 µg of protein extract was fractionated on 15% NATIVE-PAGE gels for SOD acitivty and was stained according to [10,16]

Antibodies used for immunodetection were obtained from D. Kliebenstein (University of California, Davis, CA) [17] or have been described [10] with the exception of CCS. The full length AtCCS recombinant protein was expressed in *E. coli* and purified. A polyclonal antibody was then raised in rabbit against the CCS purified protein (Pocono Rabbit Farm and Laboratory Inc, PA, USA). All antibodies were raised against *Arabidopsis* purified proteins with the exception of plastocyanin which was raised against spinach.

Transmission Electron Microscopy. Vegetative tissue from 14 day old plants were fixed in 5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 2 hours, then rinsed three times in 0.1M sodium cacodylate buffer, and post-fixed in 2% osmium tetroxide in 0.1M sodium cacodylate buffer for 2 hours. Samples were then dehydrated in acetone (30, 50, 70, 90, and 100%) for 15 minutes each. Fixation and dehydration of all plant tissue was performed at the same time and at 4°C. After which samples were brought to room temperature with the last dehydration step. Tissue was embedded over 24 hours by adding Spurr's resin, and then polymerized for 12 hours at 70°C. Thin sectioning was performed using a Porter Blum MT-2 ultramicrotome and sections were stained for 7 minutes in 1% (w/v) uranyl acetate and for 2 minutes in 0.2% (w/v) lead acetate. Thin sections were imaged using an AEI electron microscope.

3. Results

Previously, it has been demonstrated that *hma1* mutant plants exhibit yellowing of vegetative tissue when placed in high light [11]. Seeds were obtained from Norbert Rolland (Université Joseph Fourier, Grenoble, France) and propagated for this investigation. To ensure that plants contained the same phenotype that was described plants were grown for two weeks at 120 µmol photons $m^{-2} \cdot s^{-1}$ and then placed in 250 µmol photons $m^{-2} \cdot s^{-1}$ for two additional weeks. We demonstrate that the *hma1* mutant plants used for this study display the same chlorotic phenotype in high light that was previously reported (Figure 2). Chlorophyll fluorescence measurements (Fv/Fm, ΦPSII, qP, and NPQ) were taken on the soil grown mutants after high light treatment and compared to wildtype plants according to their background (Figure 3). Data recorded for PSII efficiency were significantly reduced in *hma1* mutants compared control plants; however no significance was seen in Fv/Fm measurements. When comparing *hma1* mutants to wildtype controls no trend was seen with qP and NPQ measurements that were consistent in both backgrounds.

Mutations in *PAA1* and *PAA2* result in a defect of photosynthetic activity that can be detected by measuring chlorophyll fluorescence [9,10]. If HMA1 transports copper across the envelope membrane similar to PAA1 than similar chlorophyll fluorescence phenotypes would be expected. Wildtype, *hma1*, *paa1*, and *paa2* mutant plants were grown on soil for four weeks in normal light conditions and chlorophyll fluorescence measurements were taken. In the Ws background, a significant different was displayed between *hma1* mutants (ACT and DRC) and control plants in ΦPSII and qP measurements (Figure 4). In the

Columbia background no trends were seen in Fv/Fm, ΦPSII, qP, or NPQ measurements.

When copper fed, *paa1* and *paa2*, mutants recover their growth phenotype which is most likely due to a restoration of photosynthetic activity. Since *hma1*, *paa1*, and *paa2* all exhibit significantly different PSII measurements compared to wildtype plants, we investigated whether copper feeding would restore the PSII efficiency when mutants are fed with copper. Plants were grown on ½ MS and ½ MS media supplemented with 2 μM $CuSO_4$ for 14 days in normal light. Very little change in Fv/Fm was seen between plants and between treatments (Figure 5). Photosystem II efficiency (ΦPSII) was significantly different between wildtype and all mutants in deficient and sufficient copper conditions. Interestingly, an increase in ΦPSII was observed in most plant lines when fed with copper; however copper had the least effect on *hma1* mutants (ACT, DRC, and *hma1-4*). This effect of copper feeding can be illustrated in falsely colored images of PSII efficiency where minimum value equals 0.250 (blue) and maximum value equals 0.500 (red) (Figure 6). All plant lines, with the exception of *hma1-4*, ACT, and DCR, displayed a sizable difference in photosystem II efficiency when fed copper. Most *hma1* mutant plants yielded a significantly different qP measurement in low and high copper concentrations compared to wildtype controls (Figure 7). A difference was also seen between *paa1* and wildtype in ½ MS; however, when media was supplemented with copper, qP increased in *paa1* to a level higher than control plants. Little difference was seen between *paa2* and *pc-2* plants and wildtype in treatments and did not seem to be affected by copper. Non-photochemical

quenching (NPQ) was not significantly different between Ws, ACT, and DRC plants in either treatment. Wildtype, *paa1*, *paa2*, and *pc-2* NPQ levels increased when fed with copper however *hma1* did not.

It has been previously demonstrated that mutations in *PAA1* or *PAA2* affects plastocyanin, CSD1, and CSD2 protein expression as well as SOD activity [9,10]. If HMA1 functions in copper transport for CSD2 activity in the chloroplast both expression as well as activity levels would be altered. To investigate this, wildtype (Col and Ws), *paa1*, *paa2*, *hma1*, ACT, and DRC plants were grown for 14 days on ½ MS and ½ MS supplemented with 2 μM $CuSO_4$. Wildtype controls displayed a switch between FeSOD expression and activity in low copper and Cu,ZnSOD expression and activity in sufficient copper concentrations (Figure 8). CCS and CSD1 expression in *paa1* and *paa2* plants were elevated in ½ MS media. Increased levels of CSD1 were noticeable in *paa1* plants in supplemented copper treatments, whereas expression levels of CSD2 were increased in *paa2* plants in the deficient copper treatment when compared to all other plant lines. Plastocyanin levels were dramatically affected in *paa1* and *paa2* plants in both treatments compared to controls and *hma1* mutants. In contrast to the *paa1* and *paa2* mutants, *hma1* mutants did not display a difference in CSD1, CSD2, CCS, or PC protein expression levels when compared to control plants in deficient or sufficient copper conditions. Activity of SOD followed the same trend as protein expression levels. Elevated CSD1 and CSD2 activity was seen in *paa1* and *paa2* mutants, whereas no differences in activity levels were seen between *hma1* and control plants.

Since photosynthetic activity is affected in *paa1* and *hma1* mutants that can be demonstrated by chlorophyll fluorescence (specifically PSII efficiency) this may be correlated to changes to the structure of the chloroplast. Wildtype (Col), *paa1*, and *hma1-4* were grown in ½ MS and ½ MS supplemented with 10 µM $CuSO_4$ for 14 days. Tissue was then harvested and prepared for analysis via transmission electron microscopy. We observed distinct differences in the chloroplast structure of *paa1-3* and *hma1-4* when compared to wildtype plants (Figure 9 - 11). We noted a change in the chloroplast shape in *hma1* mutants that was unlike other plant lines. Many of the chloroplasts were oblong in shape and appeared to bulge on one side of the chloroplast. In contrast, *paa1-3* mutants contained altered ultrastructure in the stroma between thylakoid membranes. A minimal amount of starch was observed in *hma1* plants, while the presence of starch in wildtype and *paa1-3* was negligible. In the sections that were imaged it appeared that the addition of copper to the media slightly decreased thylakoid stacking in all plants. However, stacking of the thylakoid membranes was the strongest in wildtype plants (Figure 12). We noted a distinct difference in organization of thylakoid membranes between *hma1* and *paa1* plant lines. A decrease in stacking was exhibited in *paa1* plants, especially in the copper treatment, whereas *hma1* plants were similar to wildtype in appearance of grana.

4. Discussion

Arabidopsis thaliana contains eight members of the HMA family that encode P_{1B}-type ATPases. Most of these genes have been characterized in

Arabidopsis, such as HMA1, HMA2, HMA4, HMA5, PAA1, RAN1, and PAA2 [9-11,18-22]. The HMA5, PAA1, RAN1, and PAA2 transporters have been demonstrated to translocate copper; whereas data on HMA2 and HMA4 suggests that their metal specificity is with zinc, cadmium, and possibly lead. The initial characterization of HMA1 suggests the function could be copper and zinc translocation. In this investigation, we have confirmed a high light phenotype of *hma1* mutants which is a yellowing of vegetative tissue. There is also a slight decrease in growth rate in these mutant plants under the same treatment. In normal light conditions *paa1* and *paa2* exhibited a moderate to severe decrease in growth rate, whereas there was no visual difference between *hma1* and wildtype control plants.

If the function of HMA1 is to transport copper into the chloroplast for CSD2 activity, protein expression as well as activity levels of the enzyme would decrease. We have reported that there is no difference in CSD2 protein levels in *hma1* mutants compared to wildtype. Furthermore, plants would also display a change in protein expression levels of CSD1 in the cytosol as well as CCS which delivers copper to CSD1 and CSD2. In *paa1* and *paa2* mutants, there is a sizable difference in expression levels of CCS, CSD1, and CSD2 proteins compared to wildtype which is most likely due to either an accumulation of copper ions in the cytosol (*paa1*) or an accumulation of copper ion in the cytosol and the stroma (*paa2*). Wildtype and *hma1* plants displayed similar CCS and CSD1 protein expression levels. Similar trends between plants were seen when SOD activity was analyzed where CSD1 and CSD2 activity were elevated in *paa1* and *paa2* and no difference was seen in

hma1 plants compared to controls. Altogether, these immunoblot and activity assays suggest that a mutation in the *HMA1* gene does not alter the affect of protein expression or activity of CSD2 or other proteins involved in copper homeostasis (CCS, CSD1, and PC) in a plant cell.

Chlorophyll fluorescence analysis is widely used to study the effects of mutations as well as treatments on photosynthetic activity. If HMA1 is involved in SOD regulation and activity within the chloroplast Fv/Fm measurements would detect possible photoinhibition as a consequence of oxidative stress caused by a defect in the transporter. However, Fv/Fm values in *hma1* mutant plants were no different than wildtype in normal light or high light treatments as well as on ½ MS media. Interestingly, an HMA1 mutation most dramatically affected PSII efficiency and qP values in all treatments that were tested. This phenotype, similar to *paa1* and *paa2*, would suggest that there is a defect in photosynthetic activity downstream of photosystem II; however a location of this defect can not be determined with this data. Since plastocyanin protein levels are severely affected in *paa1* and *paa2* it is likely that this is the location of the defect in these mutants. There is currently no experimental data to suggest where the defect is located in the photosynthetic pathway of *hma1* plants. Additionally, copper feeding affected chlorophyll fluorescence measurements in all plants except for the *hma1* mutants.

Genetic mutations of genes that encode chloroplastic proteins can cause a change in chloroplast structure, which can be severe. One such example is a mutation in chloroplastic NifS which causes a dissociation of grana and thylakoid membranes [23]. If PAA1 and HMA1 are both located in

the chloroplast envelope and have similar functions any change in the chloroplast structure would be expected to be similar between mutants. We have demonstrated a unique change in chloroplast structure in *hma1* mutants in low and high copper conditions. Here we note that there is a change in overall shape of the *hma1* chloroplasts, as a result the chloroplast appears to bulge on one side. However, we are not certain if this is the result of the mutation, fixation procedures, or if it represents the area of the leaf that was imaged. In contrast, the *paa1-3* mutant plants did not display that same change in chloroplast ultrastructure when compared to *hma1-4* plants. These plants contained stress related damage and under high copper there was a decrease in grana stacking and dissociation of thylakoids which was not as severe as NifS mutants [23].

Recently, it has been suggested that sequences within the sixth, seventh, and eighth transmembrane domains of P_{1B}-type ATPases can contribute to metal specificity [5]. In addition to metal binding and ion transduction sequences being dissimilar between HMA1 and the PAA transporters, there are also differences in the sequences within these other transmembrane domains. The PAA1 and PAA2 transporters contain xYN and MxxxS sequences in the seventh and eight transmembrane domains, respectively. In contrast, HMA1 contains a HEGG sequence in the eighth transmembrane domain. With this level a variation between HMA1 and the PAA transporter sequences it is unlikely that metal specificity for binding is the same. Topologies of PAA1 and HMA1 transporters have yet to be resolved; however, since ATP is present on both sides of the envelope it is possible for

either orientation to occur. Biochemical data suggests that PAA1 functions as an influx for copper ions in the chloroplast. It is possible that HMA1 could be orientated in the opposite direction and acts in metal efflux from the organelle. Several metals, such as cobalt and cadmium, have been shown to interact with proteins requiring copper for function. Copper, zinc superoxide dismutase enzyme crystal structure has been resolved with cadmium located in the copper binding domain [24]. Additionally, a cobalt(III) complex has been demonstrated to target the b_6f complex in photosystem II and affect photosynthetic activity [25]. If either of these two metals accumulated in the chloroplast they could have non-specific interactions with other metalloproteins and negatively affect plant physiology and biochemistry. All of the plant lines in this investigation were grown on various concentrations of copper, zinc, and cadmium in an attempt to resolve which metal HMA1 transports. However, we were unable to make conclusions on this experiment due to the two different ecotypes of the mutants that were used. It appeared that Ws and Col ecotypes of *Arabidopsis* cope with metals differently. Overall, we did not observe changes or phenotypes that were consistent cross both ecotypes in ACT, DRC, and *hma1-4* plants.

In this investigation, we report that there is little similarity between the *hma1* and *PAA* mutant plants. Although, all mutants display a chlorophyll fluorescence phenotype, especially with photosystem II efficiency, *hma1* mutants were not affected by copper feeding. In addition, CCS, CSD1, CSD2, and PC protein levels were dramatically affected in *paa1* and *paa2*, whereas there was no difference between *hma1* and wildtype controls. Superoxide

dismutase was not affected in *hma1* mutants, which was in contrast to *paa1* and *paa2*. Finally, we describe a unique chloroplast ultrastructure in *hma1* mutants that is distinctly different from wildtype or *paa1* plants. All together, we propose that the HMA1 is not a primary or secondary transporter of Cu(I) into the chloroplast for proper function of either plastocyanin or CSD2. We believe that the primary function of HMA1 is to transport a metal other than Cu(I) across the envelope membrane, which is most likely zinc but could also be cobalt or cadmium. If HMA1 does interact with copper *in planta* we believe it is a non-specific interaction. We do not believe that the function of this transporter has fully been resolved and further investigation is needed to determine the metal specificity, membrane topology, as well as the cause of defect in photosystem II efficiency.

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References

[1] Hall, J.L. and Williams, L.E. (2003). Transition metal transporters in plants. J Exp Bot 54, 2601-13.

[2] Williams, L.E., Pittman, J.K. and Hall, J.L. (2000). Emerging mechanisms for heavy metal transport in plants. Biochim Biophys Acta 1465, 104-26.

[3] Palmgren, M.G. and Axelsen, K.B. (1998). Evolution of P-type ATPases. Biochim Biophys Acta 1365, 37-45.

[4] Williams, L.E. and Mills, R.F. (2005). P1B-ATPases - an ancient family of transition metal pumps with diverse functions in plants. Trends in Plant Science 10, 491-502.

[5] Arguello, J.M., Eren, E. and Gonzalez-Guerrero, M. (2007). The structure and function of heavy metal transport P(1B)-ATPases. Biometals

[6] Axelsen, K.B. and Palmgren, M.G. (1998). Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol 46, 84-101.

[7] Axelsen, K.B. and Palmgren, M.G. (2001). Inventory of the superfamily of P-type ion pumps in Arabidopsis. Plant Physiol 126, 696-706.

[8] Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D. and Van Montagu, M. (1995). Molecular characterization of a putative Arabidopsis thaliana copper transporter and its yeast homologue. J Biol Chem 270, 28479-86.

[9] Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K.K. and Pilon, M. (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15, 1333-46.

[10] Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M. and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17, 1233-51.

[11] Seigneurin-Berny, D., Gravot, A., Auroy, P., Mazard, C., Kraut, A., Finazzi, G., Grunwald, D., Rappaport, F., Vavasseur, A., Joyard, J., Richaud, P., and Rolland, N. (2006). HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem 281, 2882-92.

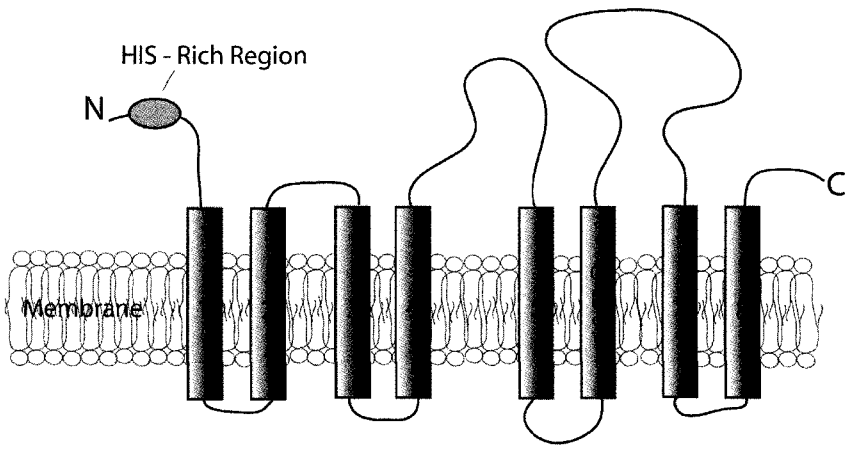
[12] Weigel, M., Varotto, C., Pesaresi, P., Finazzi, G., Rappaport, F., Salamini, F. and Leister, D. (2003). Plastocyanin is indispensable for photosynthetic electron flow in Arabidopsis thaliana. J Biol Chem 278, 31286-9.

[13] Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

[14] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., Andres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Letters 579, 2307-2312.

[15] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-54.
[16] Beauchamp, C. and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44, 276-87.
[17] Kliebenstein, D.J., Monde, R.A. and Last, R.L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118, 637-50.
[18] Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., Vavasseur, A. and Richaud, P. (2004). Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Lett 576, 306-12.
[19] Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker J.R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97, 383-93.
[20] Andres-Colas, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker, J.R., Puig, S. and Penarrubia, L. (2006). The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J 45, 225-36.
[21] Eren, E. and Arguello, J.M. (2004). Arabidopsis HMA2, a divalent heavy metal-transporting P(IB)-type ATPase, is involved in cytoplasmic Zn2+ homeostasis. Plant Physiol 136, 3712-23.
[22] Mills, R.F., Krijger, G.C., Baccarini, P.J., Hall, J.L. and Williams, L.E. (2003). Functional expression of AtHMA4, a P1B-type ATPase of the Zn/Co/Cd/Pb subclass. Plant J 35, 164-76.
[23] Van Hoewyk, D., Abdel-Ghany, S.E., Cohu, C.M., Herbert, S.K., Kugrens, P., Pilon, M. and Pilon-Smits, E.A. (2007). Chloroplast iron-sulfur cluster protein maturation requires the essential cysteine desulfurase CpNifS. Proc Natl Acad Sci U S A 104, 5686-91.
[24] Ferraroni, M., Rypniewski, W., Wilson, K.S., Viezzoli, M.S., Banci, L., Bertini, I. and Mangani, S. (1999). The crystal structure of the monomeric human SOD mutant F50E/G51E/E133Q at atomic resolution. The enzyme mechanism revisited. J Mol Biol 288, 413-26.
[25] Ceniceros-Gomez, A.E., King-Diaz, B., Barba-Behrens, N., Lotina-Hennsen, B. and Castillo-Blum, S.E. (1999). Two inhibition targets by [Cr(2gb)(3)](3+) and [Co(2gb)(3)](3+) on redox enzymes of spinach thylakoids. J Agric Food Chem 47, 3075-80.

A. HMA1 Transporter



B. PAA Transporters

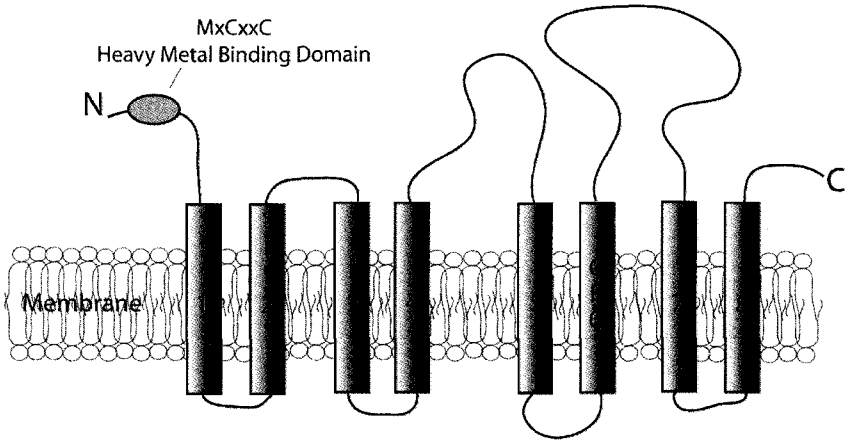


Figure 1. Models of the HMA1 and PAA transporters from *Arabidopsis thaliana*.

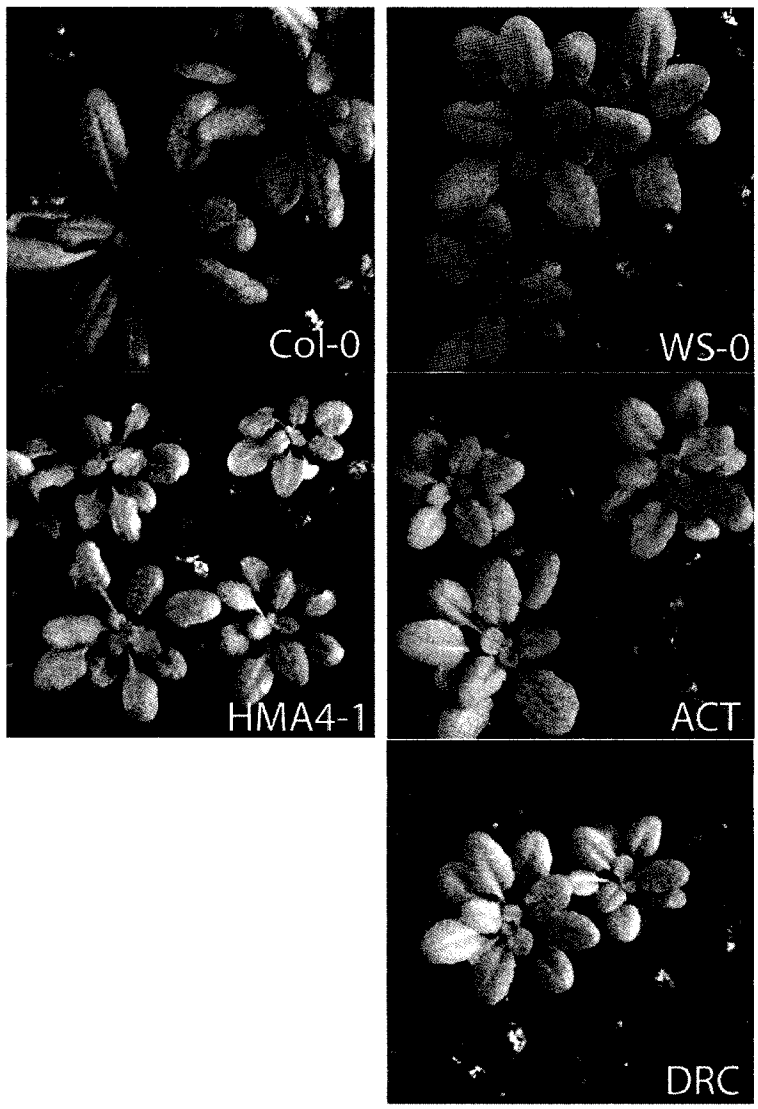


Figure 2. A mutation in *HMA1* causes yellowing of vegetative tissue in high light conditions. Images of *hma1* mutant plants and control plants grown on soil after a total of four weeks growth.

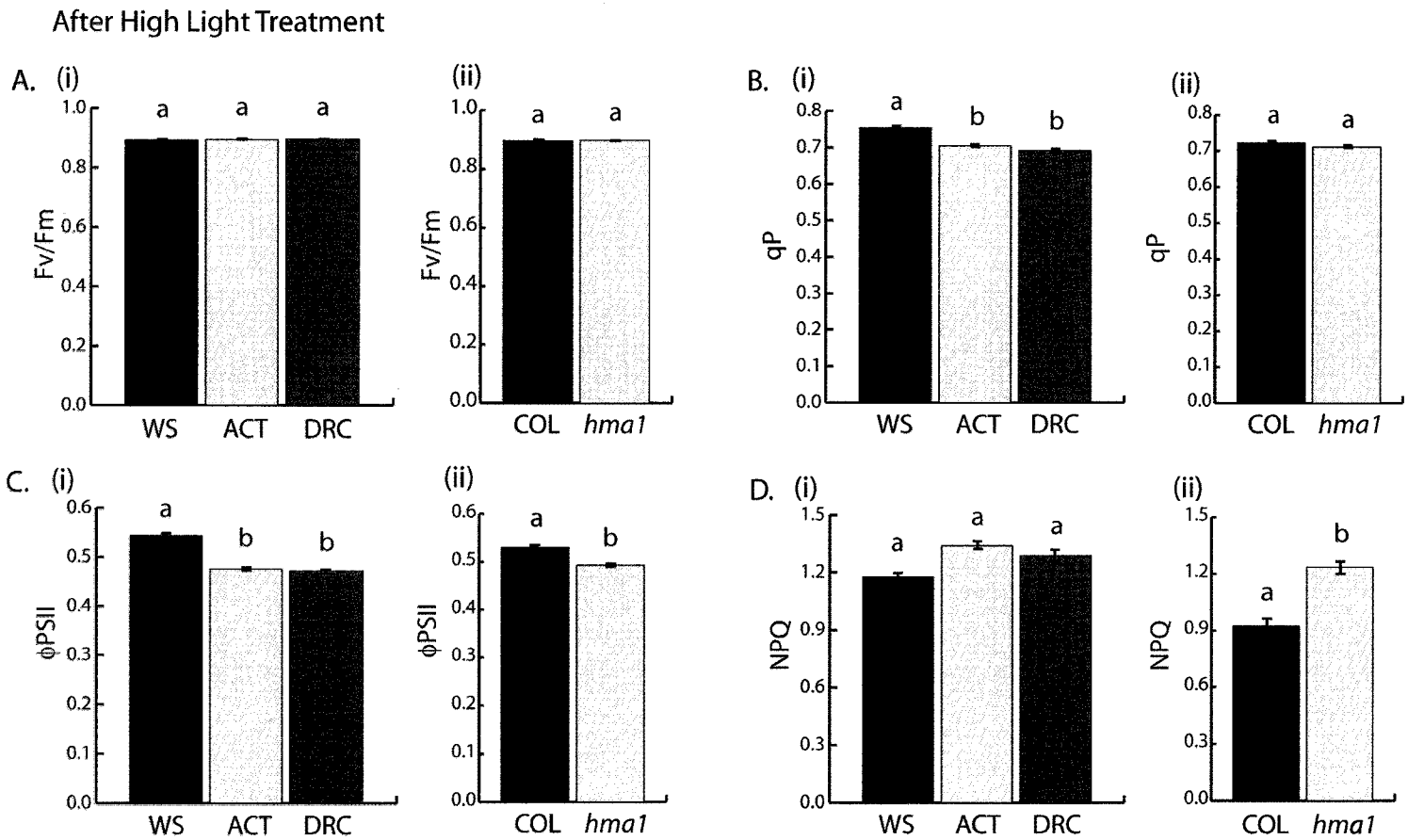


Figure 3. Measurements of mean chlorophyll fluorescence (Fv/Fm, ΦPSII, qP, and NPQ) after high light treatments ± S.E. Wildtype (Col and WS), ACT, DRC, and *hma1-4* plants were grown on soil for a total of four weeks. Letters denote significant difference of plants categorized by ecotype within treatments based on Tukey's HSD comparison of least squared means (ANOVA) (n = 2 - 6 individual measurements, p = 0.05).

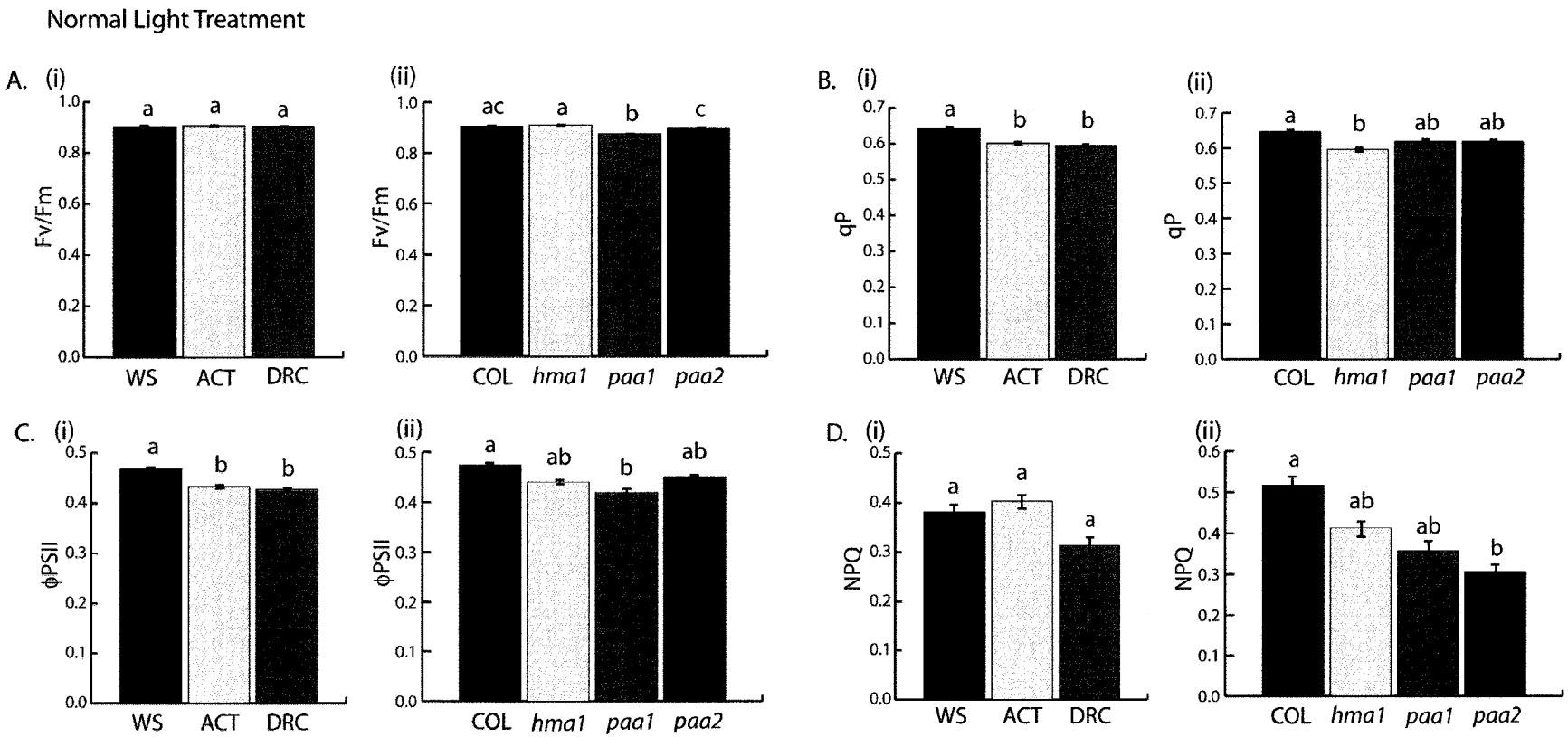


Figure 4. Measurements of mean chlorophyll fluorescence (Fv/Fm, ΦPSII, qP, and NPQ) ± S.E. Wildtype (Col and WS), ACT, DRC, *hma1-4*, *paa1-3*, *paa2-1*, and *pc-2* plants were grown on soil for a total of four weeks in normal light conditions. Letters denote significant difference between plants categorized by ecotype within treatments based on Tukey's HSD comparison of least squared means (ANOVA) (n = 4 - 8 individual measurements, p = 0.05).

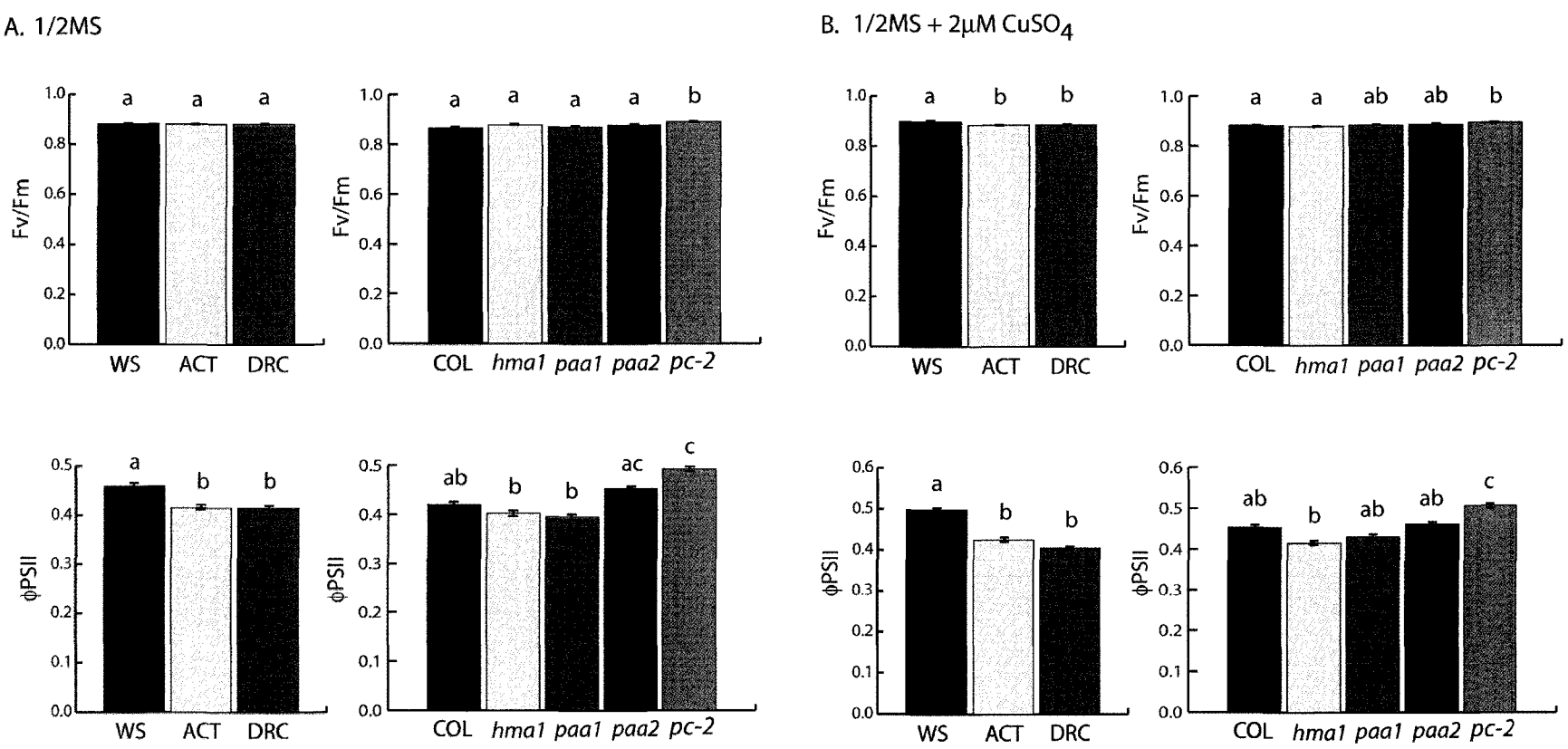


Figure 5. Measurements of mean chlorophyll fluorescence (Fv/Fm and ΦPSII) ± S.E. Wildtype (Col and WS), ACT, DRC, *hma1-4*, *paa1-3*, *paa2-1*, and *pc-2* plants were grown for 14 days on ½ MS (Panel A) or ½ MS media supplemented with 2 μM $CuSO_4$ (Panel B). Letters denote significant difference between plants categorized by ecotype within treatments based on Tukey's HSD comparison of least squared means (ANOVA) (n = 4 - 8 individual measurements, p = 0.05).

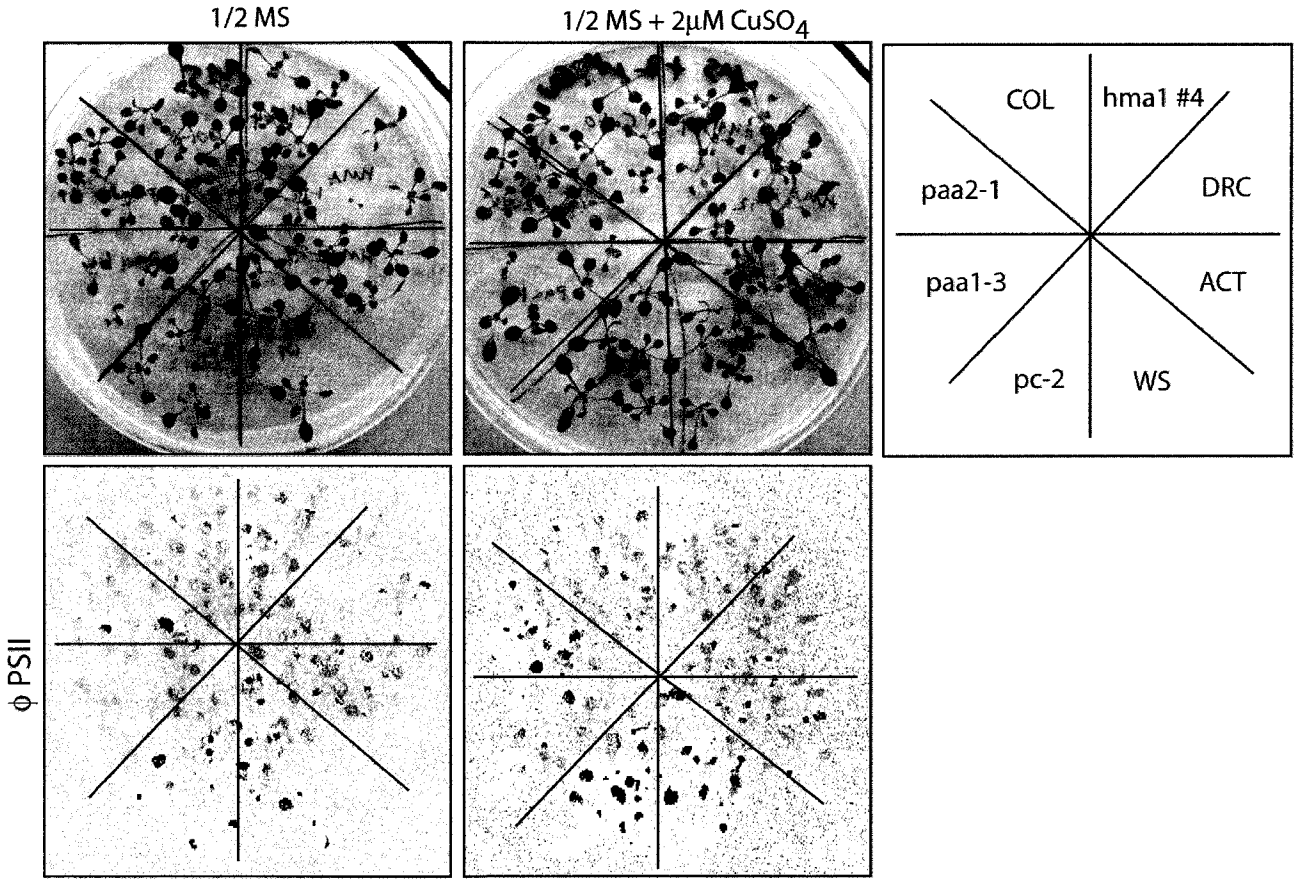


Figure 6. Images of wildtype (Col and WS), *hma1-4*, ACT, DRC, *paa1-3*, *paa2-1* and *pc-2* plants grown in ½ MS and ½ MS media supplemented with 2 μM $CuSO_4$. Plants were grown for 14 days and measured for chlorophyll fluorescence. Chlorophyll fluorescence images represent PSII efficiency values and are falsely colored (Blue, minimum value = 0.250; Red, maximum value = 0.500).

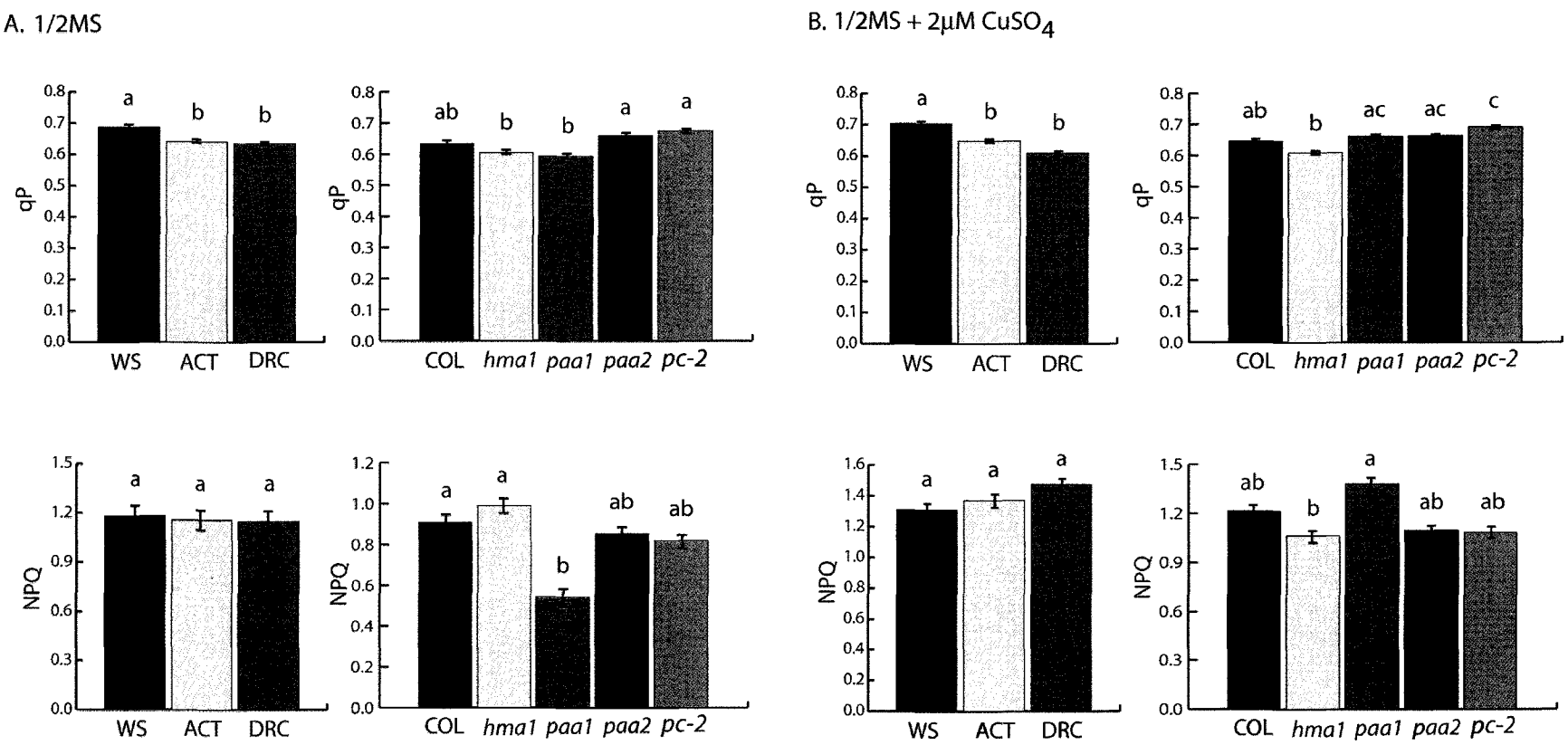


Figure 7. Measurements of mean chlorophyll fluorescence (qP and NPQ) ± S.E. Wildtype (Col and WS), ACT, DRC, *hma1-4*, *paa1-3*, *paa2-1*, and *pc-2* plants were grown for 14 days on ½ MS (Panel A) or ½ MS media supplemented with 2 μM $CuSO_4$ (Panel B). Letters denote significant difference between plants categorized by ecotype within treatments based on Tukey's HSD comparison of least squared means (ANOVA) (n = 4 - 8 individual measurements, p = 0.05).

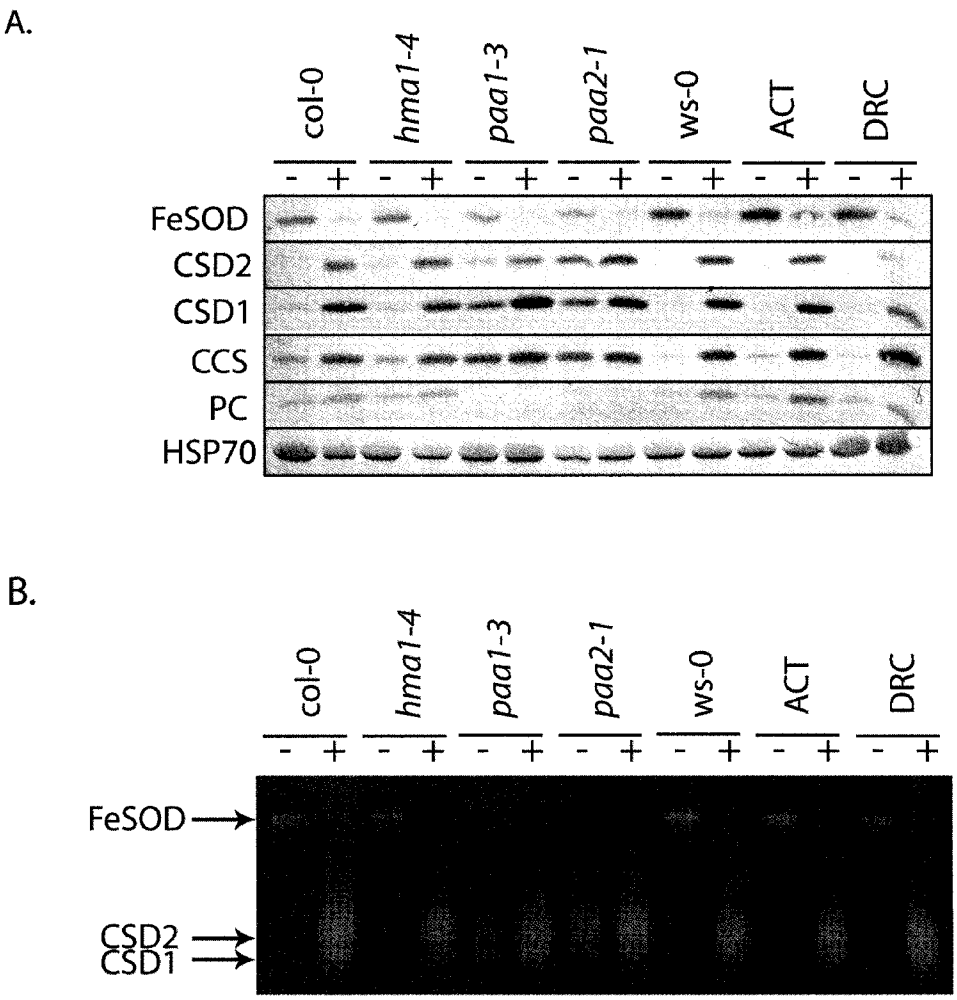


Figure 8. Altered HMA1 expression does not dramatically affect copper homeostasis protein expression or SOD activity. Immunoblot and SOD activity analysis of wildtype (Col and WS), ACT, DRC, *hma1-4*, *paa1-3*, and *paa2-1* plants grown for 14 days in ½ MS and ½ MS media supplemented with 2 μM $CuSO_4$. A total of 15 μg of soluble protein from whole plant tissue was resolved on 12.5% SDS-PAGE gels for protein expression (Panel A). HSP70 proteins were used as a loading control. A total of 10 μg of soluble protein was resolved on a 15% NATIVE-PAGE gel for SOD activity using the same plant tissue (Panel B).

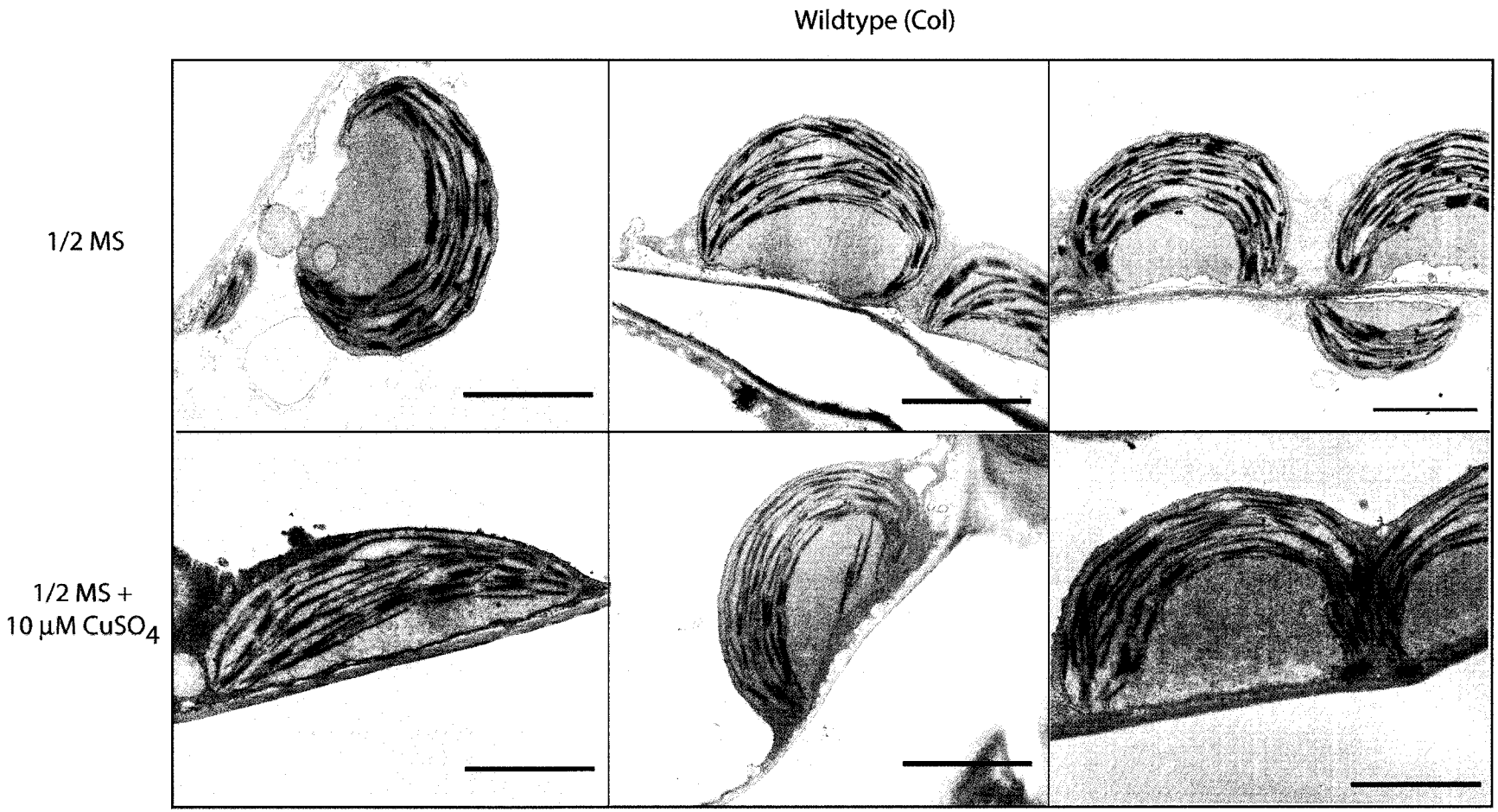


Figure 9. Chloroplast ultrastructure of wildtype (Col) plants. Wildtype (Col), *hma1-4*, and *paa1-3* plants were grown for 14 days in ½ MS and ½ MS media supplemented with 10 µM $CuSO_4$. Vegetative tissue was harvested and prepared for transmission electron microscopy analysis (Scale bars = 2 µm).

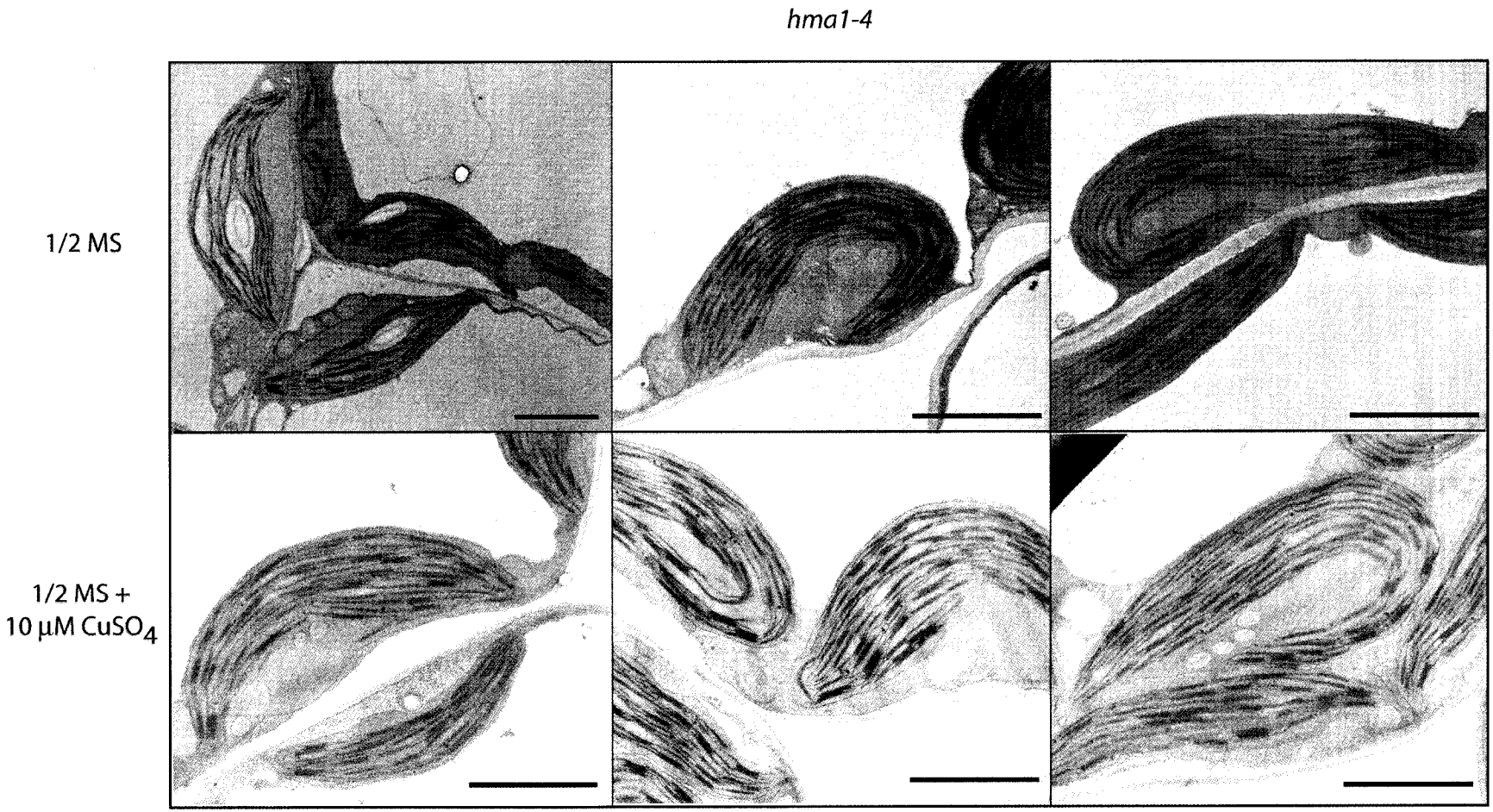


Figure 10. Chloroplast ultrastructure of *hma1-4* mutant plants. Wildtype (Col), *hma1-4*, and *paa1-3* plants were grown for 14 days in ½ MS and ½ MS media supplemented with 10 µM $CuSO_4$. Vegetative tissue was harvested and prepared for transmission electron microscopy analysis (Scale bars = 2 µm).

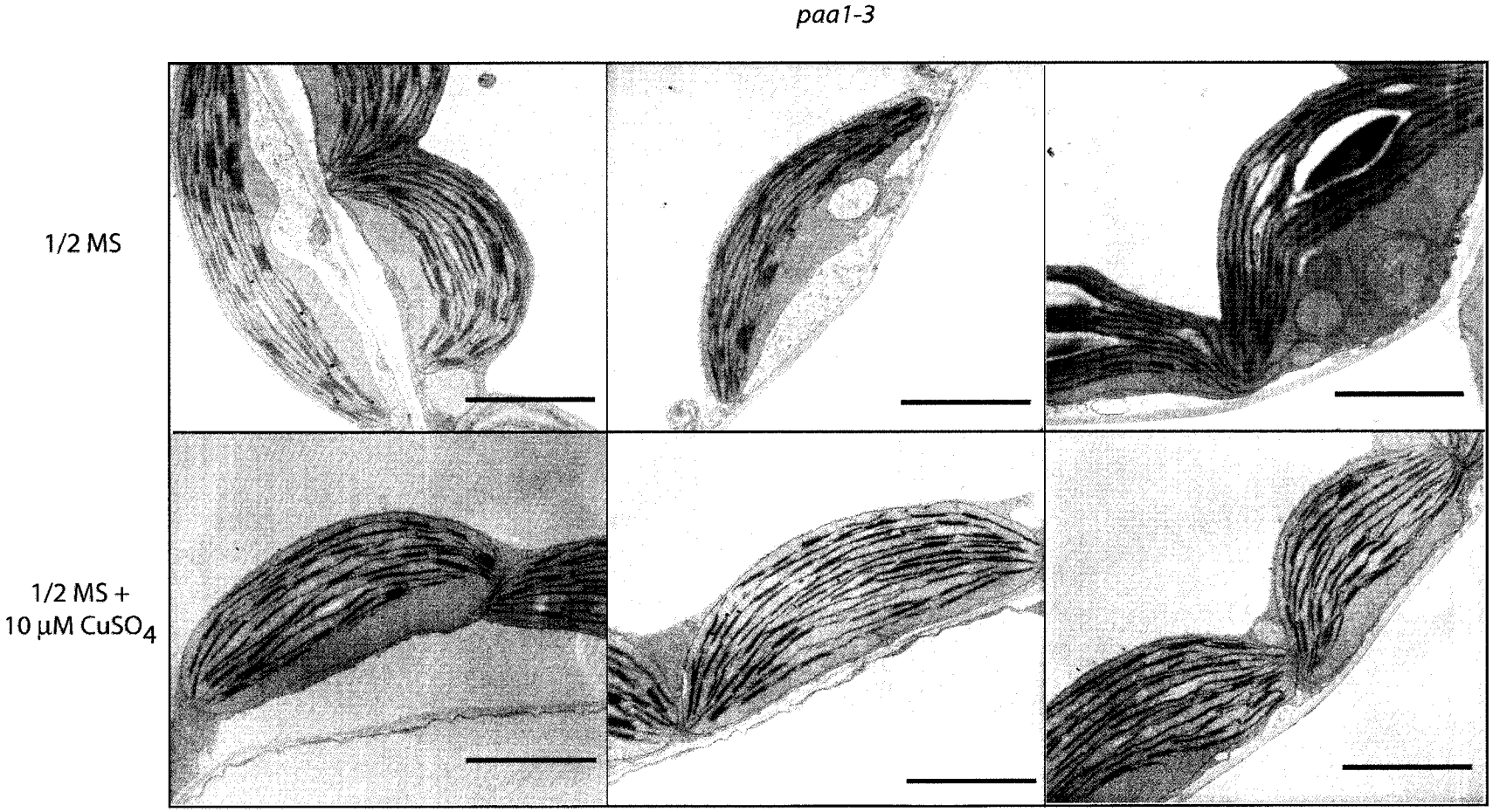


Figure 11. Chloroplast structure differs between *hma1-4* and *paa1-3* mutant plants. Micrographs of *paa1-3* mutant plants in low and high copper conditions. Wildtype (Col), *hma1-4*, and *paa1-3* plants were grown for 14 days in ½ MS and ½ MS media supplemented with 10 µM $CuSO_4$. Vegetative tissue was harvested and prepared for transmission electron microscopy analysis (Scale bars = 2 µm).

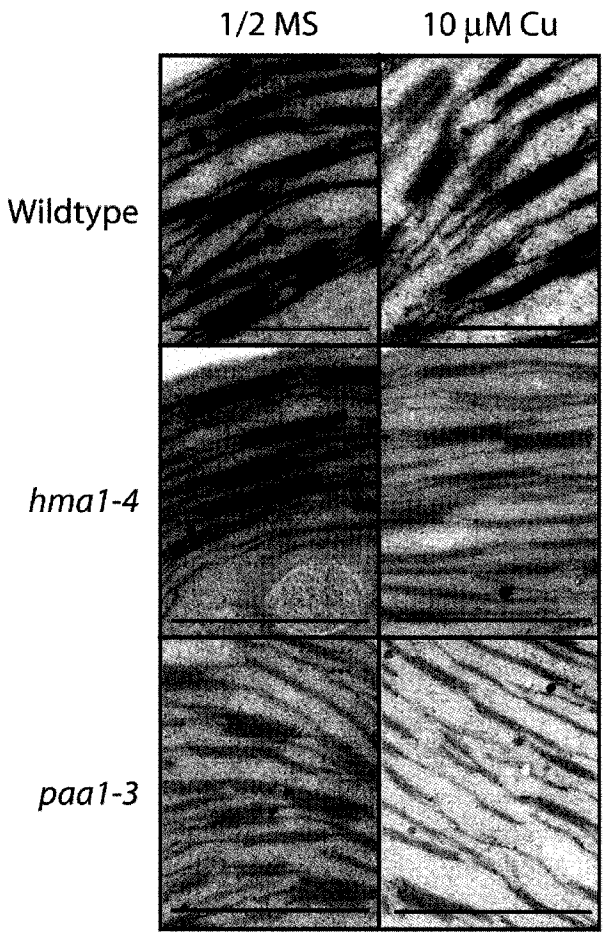


Figure 12. Magnified images of thylakoid membranes in wildtype, *hma1-4* and *paa1-3* plants. (Scale bar = 1 micron).

CHAPTER 6

A comprehensive analysis of copper transport protein-protein interactions in *Arabidopsis* studied by yeast two-hybrid

Abstract

Copper is an essential micronutrient for plants and plays a crucial role in processes such as, photosynthesis and reactive oxidative species detoxification. Since excess copper is toxic there is a balance between the need for copper and copper toxicity that the plant has to achieve in order to survive. Due to its extreme toxicity, copper must be tightly regulated by the cell. This regulation involves proteins that can carry copper (called copper chaperones) as well as specific copper transporters that shuttle copper across membranes. Copper is an essential micronutrient, yet copper delivery is not well understood in plants. In this investigation, protein-protein interactions for a total of 65 different combinations were tested in the yeast two-hybrid system to look for new possible transport pathways for copper in a plant cell. Special interest was taken in finding candidate proteins for the transport of copper to the chloroplast and within the chloroplast to the thylakoid membrane for plastocyanin. Several new possible copper delivery pathways were identified *in vitro*. Most interestingly, and plausible *in vivo*, an interaction was observed

between the CCS chaperone and the amino terminal domain of HMA5 suggesting that CCS could aid in cell detoxification or HMA5 could donate copper to CCS for delivery to CSD1. Also an interaction was determined between the amino terminal domains of PAA1 and PAA2 suggesting a possible direct pathway for copper within the chloroplast.

Abbreviations used:

HMA1(N)	Amino terminal domain of the Arabidopsis HMA1 protein without a transit peptide
HMA4(N)	Amino terminal domain of the Arabidopsis HMA4 protein
HMA5(N)	Amino terminal domain of the Arabidopsis HMA5 protein
PAA1(N)	Amino terminal domain of the Arabidopsis PAA1 protein without a transit peptide
PAA2(N)	Amino terminal domain of the Arabidopsis PAA2 protein without a transit peptide
ATX1	Full length ATX1 protein from Arabidopsis
CCH	Full length CCH protein from Arabidopsis
CCHΔ	CCH protein from Arabidopsis with a deletion of the C-terminus
CCS	Full length CCS protein from Arabidopsis without transit peptide
CCP	Full length CCP protein from Arabidopsis without transit peptide
CCP#2	CCP protein from Arabidopsis with an N-terminal deletion (based on an alternative cleave site of mature protein)
CutA	Full length CutA protein from Arabidopsis without transit peptide

1. Introduction

Plants require the transport of essential micronutrients, like copper, long distance through organs and across several membranes before they reach their final destination in target enzymes. Membrane transporters and chaperones are the proteins involved in cellular copper transport in plants. *Arabidopsis* contains two different transporter families responsible for the translocation of copper ions across membranes. The Copper Transporter Family (COPT) is similar to a yeast family of copper transporters (Ctr) which are all predicted to have three transmembrane domains [1,2]. Copper enters the plant cell by COPT1[3], and is either sequestered or trafficked to targets by

copper chaperones. The Heavy Metal Associated (HMA) Family of ion transporters is classified in the P_{1B}-type ATPase subfamily. Within the HMA family, HMA5, PAA1 (HMA6), RAN1 (HMA7), and PAA2 (HMA8) all contain MxCxxC heavy metal binding domains at the amino terminus. The HMA5 protein is hypothesized to be in the plasma membrane and functions in copper detoxification [4], while the RAN1 (Responsive-to-antagonist1) transporter is responsible for translocation of copper into the endomembrane system were it is required for ethylene signaling [5]. Both RAN1 and HMA5 transporters contain two separate MxCxxC domains in their amino terminus. The PAA1 and PAA2 proteins are functional homologs of the cyanobacterial transporters, CtaA and PacS, and have been localized to the chloroplast envelope and thylakoid membrane, respectively [6,7]. The HMA1 transporter has a long histidine rich region in the amino terminus that is a characteristic of other zinc transporters in the Heavy Metal Associated Family; however more recent experimental data suggests that HMA1 may function as an alternate copper transporter in the chloroplast envelope [8].

In addition to transporters, organisms also have copper chaperones to shuttle Cu(I) from one place to another within the cell. Copper chaperones are small, soluble, intracellular proteins that carry Cu(I) from one target to another and it is believed that copper chaperones are highly target-specific [9]. *Arabidopsis* contains a copper chaperone for superoxide dismutase (CCS) that has been localized to both the cytosol and the stroma and functions in delivering copper to CSD1 and CSD2 [10,11]. Additionally, there are two other cytosolic chaperones that have been identified, ATX1 and CCH, which are

both similar in structure and function to the yeast chaperone ATX1[12,13]. Previous investigations have shown that both of these chaperones interact with the amino termini of HMA5 and RAN1 [4,13].

Several other putative copper related proteins have been identified in *Arabidopsis* that may function as copper chaperones as well. The protein, CCP (Copper Chaperone for the Plastid), is similar in structure to the yeast ATX1 chaperone; however CCP contains an IxCxxC motif rather than the typical metal binding domain (MxCxxC). The CCP protein has a long series of serine repeats and the function of the protein is currently unknown [14]. This chaperone has been localized to the chloroplast [14] and one hypothesis is that it could be the chaperone for plastocyanin. A second putative copper chaperone, CutA, contains a predicted transit sequence for the chloroplast [15]. It is expressed in all major plant tissues at similar levels and purified recombinant protein of the *Arabidopsis* CutA has been shown to bind Cu(II) at a level of nearly one mole copper per one mole of protein [15].

The chloroplast is a complex organelle which has to communicate with the cell in order to acquire sufficient copper for photosynthetic activity. In this investigation, we utilize the yeast two-hybrid technique to investigate possible interactions between components of copper delivery pathways within *Arabidopsis*. Here, we describe two novel interactions between PAA1(N) and PAA2(N), as well as HMA5(N) and CCS and propose a new model for copper transport within the cell. We feel these interactions are likely *in planta* based on previously reported protein localizations and further investigations can be conducted to test these new putative transport pathways.

2. Materials and Methods

In silico sequence analysis. Gene and protein information was obtained through The Arabidopsis Information Resource (TAIR) database. Information about proteins in this study including gene loci can be found in Table 1. Chloroplast targeting sequences and potential transit peptide cleavage sites were predicted using the ChloroP Server [16]. Due to a large region of serine repeats that is typically found in transit sequences, a putative shorter version of the mature protein CCP, CCP#2, was used in this investigation. Sequence alignments were performed using the CLUSTAL W (1.83) multiple sequence alignment tool [17].

Table 1. Protein Information for yeast two-hybrid analysis.

Protein Name	Gene Locus	Protein Length	Predicted Transit Peptide	Cloned Region
HMA1	AT4G37270	820 aa	Yes (Chloroplast)	61-124 aa
HMA4	AT2G19110	1173 aa	No	1-100 aa
HMA5	AT1G63440	996 aa	No	1-289 aa
PAA1	AT4G33520	950 aa	Yes (Chloroplast)	30-157 aa
PAA2	AT5G21930	884 aa	Yes (Chloroplast)	67-179 aa
ATX1	AT1G66240	106 aa	No	1-106 aa
CCH	AT3G56240	121 aa	No	1-121aa,
CCHΔ				1-69 aa
CCS	AT1G12520	320 aa	Yes (Chloroplast)	68-320 aa
CCP	AT2G28660	265 aa	Yes (Chloroplast)	84-265 aa,
CCP#2				180-265 aa
CutA	AT2G33740	182 aa	Yes (Chloroplast)	71-182 aa

Bacteria and yeast cell strains. Escherichia coli strain DH5α (F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) was used for cloning and propagation of plasmids and was cultured in Luria-Bertani (LB) broth Miller (EMD Biosciences) medium with the appropriate antibiotic. *Saccharomyces cerevisiae* strain AH109 (*MATa, trp1-901, leu2-3,*

112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, MEL1GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ) [18] was obtained from Clontech Laboratories, Inc. (Mountain View, CA, USA) and was cultured in yeast extract/peptone/dextrose (YPD) (1% (w/v) yeast extract (Fisher Scientific), 2% (w/v) peptone (Fisher Scientific), 2% (w/v) D-glucose (Fisher Scientific)) medium or synthetic complete drop out (SD) medium (Difco) lacking tryptophan and leucine for the selection and maintenance of plasmids.

Plasmid constructs for yeast two-hybrid system. Oligonucleotide primer sequences used to generate DNA fragments and sequencing of plasmids are found in Table 2. Amplification of DNA encoding N-terminal domains of HMA1, HMA4, PAA1, PAA2, full length sequences of CCS, CCP, CutA, and a truncated version of CCP were performed by PCR using Expand High Fidelity polymerase (Roche). Genomic DNA and/or cDNA isolated from wildtype *Arabidopsis* plants were used as templates for fragment amplification. All coding regions were cloned in frame into pGBKT7 and/or pGADT7 vectors (Matchmaker™ Yeast Two-Hybrid System 3, Clontech) using the *Bam*HI or *Nde*I/*Bam*HI restriction site(s).

Table 2. Primer sequences for yeast two-hybrid constructs.

Name	Direction	Oligonucleotide Primer Sequence (5'-3')
HMA1(N)	Forward	GGGAATTCCATATGCTACGTGCTGTCGAAGAT
	Reverse	CGCGGATCCCTCTCTGAGGTAATTGGCCAA
HMA4(N)	Forward	GGGAATTCCATATGGCGTTACAAAACAAAGAAGAA
	Reverse	CGCGGATCCCGAAAGGGCTCGGCCATTTGTTCT
PAA1(N)	Forward	GGGAATTCCATATGTCGGAGAGTGGTGATTCCAAGTCA
	Reverse	CGCGGATCCGCTCGCGGCCACTCTCTTTTAAGCG
PAA2(N)	Forward	GGGAATTCCATATGATCGAATCTGTGAAATCCATTACGA
	Reverse	CGCGGATCCCACGGTTCCTGCTCTTAACAAGCAA
CCS	Forward	GGGAATTCCATATGGCGACTGCTCTCACTTC

	Reverse	ATAGGAATGCGGCCGCGGATCCTTAAACCTTACTGGCCACGAAA
CCP	Forward	GGGAATTCCATATGGCTTCGGCTCGCGGTAGC
	Reverse	ATAGGAATGCGGCCGCGGATCCTTAAGATTTGAGGAGTGAATAAT
CCP#2	Forward	GGGAATTCCATATGACTGATGACCAGGTTGTT
	Reverse	ATAGGAATGCGGCCGCGGATCCTTAAGATTTGAGGAGTGAATAAT
CutA	Forward	GGGGTACCATGGAGGAGAGCAGCAAAACTG
	Reverse	GGGGTACCGGATCCTGGTCCAGTATTACACTTCACA
pGAD	Forward	CGATGATGAAGATACCCCAACCAAACCA
	Reverse	CAGTTGAAGTGAACTTGCGGGGTTTTTCA
pGBK	Forward	CATCATCGGAAGAGAGTAGTAACAAAGGTCA
	Reverse	CTACAGGAAAGAGTTACTCAAGAATAAGAATTTTCGT

Additional sequences were introduced to create and properly cleave restriction sites. These sequences are denoted with an underline.

The pGBK-HMA5(N), pGAD-ATX1, pGAD-CCH, and pGAD-CCHΔ plasmids were obtained from Sergi Puig (Departament de Bioquímica i Biologia Molecular, Universitat de València, Spain) and are described [4].

DNA sequence analysis. Plasmid DNA was sequenced in two directions using the forward and reverse gene specific primers used in cloning and/or the forward and reverse plasmid specific primers. Sequencing was performed by Macromolecular Resources (Colorado State University, Fort Collins, CO, USA).

Yeast two-hybrid spot assays. Yeast cells were simultaneously co-transformed with a plasmid containing an activation domain fusion and one containing a binding domain fusion using the lithium acetate (LiAc) method [19]. Selection of the transformants was performed on 1.5% agar plates lacking tryptophan and leucine and several individual colonies were purified for spot assays. Liquid cultures of yeast co-transformed with cloned pGAD and pGBK based vectors were propagated in the proper medium for selection of both plasmids

for 24 hours at 30° C, shaking at 260 RPM. The optical densities of yeast cultures were measured at 600nm using a DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter, Inc.) and diluted to 0.1 OD_{600}, then further diluted to 0.01 OD_{600} for spot assays. An 8-channel pipettor was used to transfer 3 µL of each dilution onto three separate agar plates containing SD minimal medium lacking tryptophan, leucine or medium lacking tryptophan, leucine, histidine, and adenine or medium lacking tryptophan, leucine, histidine, and adenine supplemented with 2.5 mM 3-amino-1,2,4-triazole (Sigma). Agar plates were then incubated in 30° C for five days and each assay was repeated in at least three independent experiments.

Yeast two-hybrid β-galactosidase activity assays. The procedure for quantitative estimates of yeast two-hybrid interactions using β-galactosidase was modified from the Yeast Protocol Handbook (Clontech) [19]. Liquid cultures of yeast co-transformed with cloned pGAD and pGBK vectors were propagated in the proper dropout medium for selection of both plasmids for 24 hours at 30° C, shaking at 260 RPM. Yeast extract/peptone/dextrose medium was added to each culture tube and incubated in the same conditions for an additional five hours. Liquid β-galactosidase activity assays were then carried out following the Clontech protocol using chlorophenol red-β-D-galactopyranoside (CPRG) (BMCC) as the substrate [19]. Each culture was measured in triplicate for an individual experiment and four independent experiments were performed. β-galactosidase Miller units [20,21] were calculated and quantities are presented as average values ± mean standard

deviations for the four experiments combined. Statistical analysis was performed using the Jump-in software package (SAS Institute, Cory, NC).

3. Results

In this investigation, we utilized the yeast two-hybrid technique to screen for interactions between metalloproteins in order to establish new delivery pathways for copper within *Arabidopsis*. A total of 65 different combinations of protein-protein interactions were tested including five different P_{1B}-type ATPase transporters in the HMA family (HMA1, HMA4, HMA5, PAA1, and PAA2). When all five transporter domains were aligned, sequence analysis reveals little similarity between the amino terminal domains of these transporters (Figure 1). However, previous phylogenetic investigations have shown that PAA1 and PAA2 are very similar in sequence and both proteins are similar in sequence to HMA5 [22]. This sequence similarity is most likely in the ATX-like domains of the amino termini. Additionally, there was little similarity between all five copper chaperones that were incorporated to this investigation, ATX1, CCH, CCP, CCS, and CutA (Figure 2). Although little sequence similarity exists between all proteins in this investigation, individually each protein contains either a putative metal bind domain, is predicted to be structurally similar to other copper transport proteins, or have experimentally been shown to bind copper.

The transporter domains in this study were divided into two groups based on the predicted metal ion that they transport. Putative copper transporters, HMA5, PAA1, and PAA2, were placed into one group and HMA1

and HMA4 were designated to the zinc transport group. Primarily HMA4 assays were conducted as negative controls for the copper protein assays; whereas HMA1 was included to possibly resolve what metal the protein transports. Three separate assays were performed to test interactions between these two groups of transporters (Figure 3). Interactions were observed between the copper transporters PAA1(N) and PAA2(N) as well as HMA5(N) and PAA2(N). Due to the recent experimental data that suggests that HMA1 can possibly transport both copper and zinc ions an assay was conducted to determine whether interactions could occur between transporters in the copper group and the zinc group. Interactions were seen between PAA1(N) and HMA1(N) in addition to HMA5(N) and HMA1(N). A final transporter-transporter assay was conducted on the zinc transporters, HMA4 and HMA1, in which no interaction was observed.

In order to determine potential candidate chaperones for copper transport into and within the chloroplast transporter-chaperone assays were conducted with the amino terminal domains of PAA1 and PAA2 (Figure 4). Surprisingly, no interaction was seen with both transporters and any of the candidate chaperones that were tested. Other possible cytosolic copper transport pathways were investigated with HMA5 and interactions were observed between HMA5(N) and the chaperones ATX1, CCHΔ, and CCS (Figure 5). The zinc transporters, HMA1(N) and HMA4(N), were tested with copper chaperones in the yeast two-hybrid system as negative controls. As expected, we observed no interaction between these zinc transporters and the copper chaperones (Figure 6). The CutA protein was added at a later date in

this investigation and when tested with the amino termini of all transporters no interaction was observed (Figure 7).

It might be possible for copper chaperones to act in concert with other chaperones passing ions to ensure the metal is delivered to the most important target enzymes, such as plastocyanin. To test this idea, two chaperone-chaperone assays were conducted with CCS and CutA. In both instances no interaction was seen between CCS or CutA and other chaperones. However, it was determined that CCS and CutA both interact with themselves (Figure 8).

It appeared on yeast two-hybrid spot assays that there were varying levels of interaction based on the magnitude of yeast growth on agar plates. A β-galactosidase activity assay was performed in order to gain a better estimate of the strength of interactions that were observed on these spot assays. The three interactions PAA1(N)/PAA2(N), HMA5(N)/PAA2(N), and HMA5(N)/CCS were dramatically stronger than any other interaction that was tested (Figure 9). These interactions were over 3-fold stronger than HMA5(N)/HMA1(N) which was the next strongest interaction.

4. Discussion

Previous research has shown that the yeast two-hybrid technique is a useful tool for investigating protein-protein interactions within cellular copper transport pathways in bacteria, yeast, humans, and plants [4,13,23,24]. The research presented here identified ten total positive protein-protein interactions

(Figure 10). However, not all of these interactions would be plausible in a plant cell.

Crystal structure analysis of the CutA protein isolated from *Thermotoga maritima* has demonstrated that the protein is dynamic and can exists as a homotrimer [25]. In addition, the crystal structure of the yeast CCS protein has resolved that it occurs as a homodimer [26]. Therefore, this natural trimerization and dimerization of these proteins would explain the CutA/CutA and CCS/CCS interactions that were seen in this investigation.

As stated earlier, it has been shown that the amino terminus of HMA5 can interact with the ATX1 chaperone as well as the CCH chaperone (with a C terminal deletion) [4]. These interactions were reconfirmed here although the interactions were not as strong (yeast growth on plates) as the previous publication demonstrated. One explanation for this is the minimal media that was used in this investigation compared to dropout media that may have been used in any previous investigation. The dropout medium would contain additional amino acids that would aid in the growth of yeast. Additionally, an increased amount of the inhibitor 3-amino-1,2,4-triazole was used in this study. As a result of potential differences in procedure we cannot compare between investigations.

A new interaction was discovered between the amino terminus of HMA5 and the CCS copper chaperone. P_{1B}-type ATPase transporters, such as HMA5, are predicted to have eight transmembrane domains. Other characteristics include an ATP binding site in a large loop between the sixth and seventh transmembrane domains. Since ATP is required for transport to

occur, it is believed that the location of the large loop that contains the ATP binding domain is in the cytosol. Therefore, the HMA5 terminal domains would also be orientated in the cell cytosol. Since CCS has been localized to both the cytosol as well as the stroma [11,27], it is plausible that in a plant cell the cytosolic version of CCS would have access to interact with the amino terminal domain of HMA5. If HMA5(N) and CCS do interact in the plant cell, several different mechanisms are possible. Hypotheses include that CCS could acquire excess copper from HMA5(N) for delivery to CSD1 or more likely CCS might aid in cell detoxification by delivering copper to HMA5(N) as well as CSD1.

It was expected that no interaction would be observed between the putative zinc transporters, HMA1 and HMA4, and copper chaperones. However, it was surprising that no interactions existed between PAA1 or PAA2 and the copper chaperones that were tested. As a result, there is still currently no candidate copper chaperone for transport to the chloroplast envelope.

Currently, there has been no data to suggest that P-type ATPases could interact with one another or transfer metal directly from transporter to transporter. The hypothesis for copper delivery has been such that copper chaperones transport metal from one transporter to another in a highly specific manner. This investigation has demonstrated that it is possible for P-type ATPases to interact with one another at their amino termini if cellular localization and protein orientation permits. The interaction shown between HMA5(N) and PAA2(N) would be unlikely in a plant cell due to the localization of the two proteins in the cell membrane and thylakoid, respectively. Although

it has not been definitively determined what metal HMA1 transports, characteristically it is more similar to other zinc transporters in the same subfamily. The interactions that were seen between PAA1(N) and HMA1(N) as well as HMA5(N) and HMA1(N) would not be likely if one is a copper transporter and one is a zinc transporter regardless of their locations and orientations. One interaction that is possible in the plant cell is the interaction between the amino termini of PAA1 and PAA2. Interestingly, there is sequence similarity between the copper chaperone ATX1 and the amino termini of some P_{1B}-type ATPases, such as PAA1 and PAA2. Therefore, these domains are predicted to have a βαββαβ ATX-like fold which is characteristic of many copper chaperones. One interesting hypothesis to consider is that the amino terminal domains of P_{1B}-type ATPases could act as their own chaperone for direct transport from one transporter to another.

Generally, protein-protein interactions can be affected by three characteristics of the individual proteins: charge, size, and conformation. A broad trend was seen in transporter-transporter interactions when analysis was done on the relative charge of amino terminal domains. It seemed that charge, at least in part, was responsible for interactions between these proteins and could be the reason that the amino termini of transporters did not interact with themselves. This trend was not seen in the interactions between HMA5(N) and the chaperones, CCS-CCS, or CutA-CutA. Therefore, these interactions might be based more on size and conformation of proteins rather than charge.

In this study several new copper transport protein-protein interactions were discovered through yeast two-hybrid analysis. However, not all interactions seen *in vitro* are possible within the plant cell. This screening yielded two potential new transport pathways for copper in plants (Figure 11). An interaction between HMA5(N) and CCS would be possible *in vivo* due to the cellular locations of both proteins and most excitingly, an interaction was shown between PAA1(N) and PAA2(N). Depending on the topology of these two transporters in their respective membranes (chloroplast envelope and thalykoid) copper could be directly passed from transporter to another. To date, there has been no evidence that P-type ATPase transporters could interact with other P-type ATPases, however through this investigation it has been shown that these transporters can interaction with each other at the amino terminus region of both proteins. Further research should be continued on these two new potential transport pathways for copper within a plant cell.

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References

[1] Sancenon, V., Puig, S., Mira, H., Thiele, D.J. and Penarrubia, L. (2003). Identification of a copper transporter family in Arabidopsis thaliana. Plant Mol Biol 51, 577-87.

[2] Harris, E.D. (2000). Cellular copper transport and metabolism. Annu Rev Nutr 20, 291-310.

[3] Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D. and Van Montagu, M. (1995). Molecular characterization of a putative Arabidopsis thaliana copper transporter and its yeast homologue. J Biol Chem 270, 28479-86.

[4] Andres-Colas, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker, J.R., Puig, S. and Penarrubia, L. (2006). The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J 45, 225-36.

[5] Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker J.R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97, 383-93.

[6] Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K.K. and Pilon, M. (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15, 1333-46.

[7] Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M. and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17, 1233-51.

[8] Seigneurin-Berny, D., Gravot, A., Auroy, P., Mazard, C., Kraut, A., Finazzi, G., Grunwald, D., Rappaport, F., Vavasseur, A., Joyard, J., Richaud, P., and Rolland, N. (2006). HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem 281, 2882-92.

[9] Harrison, M.D., Jones, C.E., Solioz, M. and Dameron, C.T. (2000). Intracellular copper routing: the role of copper chaperones. Trends Biochem Sci 25, 29-32.

[10] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., Andres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Letters 579, 2307-2312.

[11] Chu, C.C., Lee, W.C., Guo, W.Y., Pan, S.M., Chen, L.J., Li, H.M. and Jinn, T.L. (2005). A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. Plant Physiol 139, 425-36.

[12] Himelblau, E., Mira, H., Lin, S.J., Culotta, V.C., Penarrubia, L. and Amasino, R.M. (1998). Identification of a functional homolog of the yeast copper homeostasis gene ATX1 from Arabidopsis. Plant Physiol 117, 1227-34.

[13] Puig, S., Mira, H., Dorcey, E., Sancenon, V., Andres-Colas, N., Garcia-Molina, A., Burkhead, J.L., Gogolin, K.A., Abdel-Ghany, S.E., Thiele, D.J., Ecker, J.R., Pilon, M., Penarrubia, L. (2007). Higher plants possess two different types of ATX1-like copper chaperones. Biochemical and Biophysical Research Communications 354, 385-390.
[14] Burkhead, J. and Colorado State University. Dept. of Biology. (2003) Copper traffic in plants : roles for newly isolated chloroplast proteins, pp. 145. Colorado State University, Fort Collins, CO.
[15] Burkhead, J.L., Abdel-Ghany, S.E., Morrill, J.M., Pilon-Smits, E.A. and Pilon, M. (2003). The Arabidopsis thaliana CUTA gene encodes an evolutionarily conserved copper binding chloroplast protein. Plant J 34, 856-67.
[16] Emanuelsson, O., Nielsen, H. and von Heijne, G. (1999). ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 8, 978-84.
[17] Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G. and Thompson, J.D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31, 3497-500.
[18] James, P., Halladay, J. and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425-36.
[19] Clontech. (2001) Yeast Protocols Handbook. Mountain View, CA.
[20] Miller, J.H. (1972) Experiments in Molecular Geneticsed.^eds). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[21] Miller, J.H. (1992). In A Short Course in Bacterial Genetics ed.^eds), pp. 74. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[22] Williams, L.E. and Mills, R.F. (2005). P1B-ATPases - an ancient family of transition metal pumps with diverse functions in plants. Trends in Plant Science 10, 491-502.
[23] Lim, C.M., Cater, M.A., Mercer, J.F. and La Fontaine, S. (2006). Copper-dependent interaction of dynactin subunit p62 with the N terminus of ATP7B but not ATP7A. J Biol Chem 281, 14006-14.
[24] van Dongen, E.M., Klomp, L.W. and Merkx, M. (2004). Copper-dependent protein-protein interactions studied by yeast two-hybrid analysis. Biochem Biophys Res Commun 323, 789-95.
[25] Savchenko, A., Skarina, T., Evdokimova, E., Watson, J.D., Laskowski, R., Arrowsmith, C.H., Edwards, A.M., Joachimiak, A., and Zhang, R.G. (2004). X-ray crystal structure of CutA from Thermotoga maritima at 1.4 A resolution. Proteins 54, 162-5.
[26] Lamb, A.L., Wernimont, A.K., Pufahl, R.A., Culotta, V.C., O'Halloran, T.V. and Rosenzweig, A.C. (1999). Crystal structure of the copper chaperone for superoxide dismutase. Nat Struct Biol 6, 724-9.
[27] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., Andres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Lett 579, 2307-12.

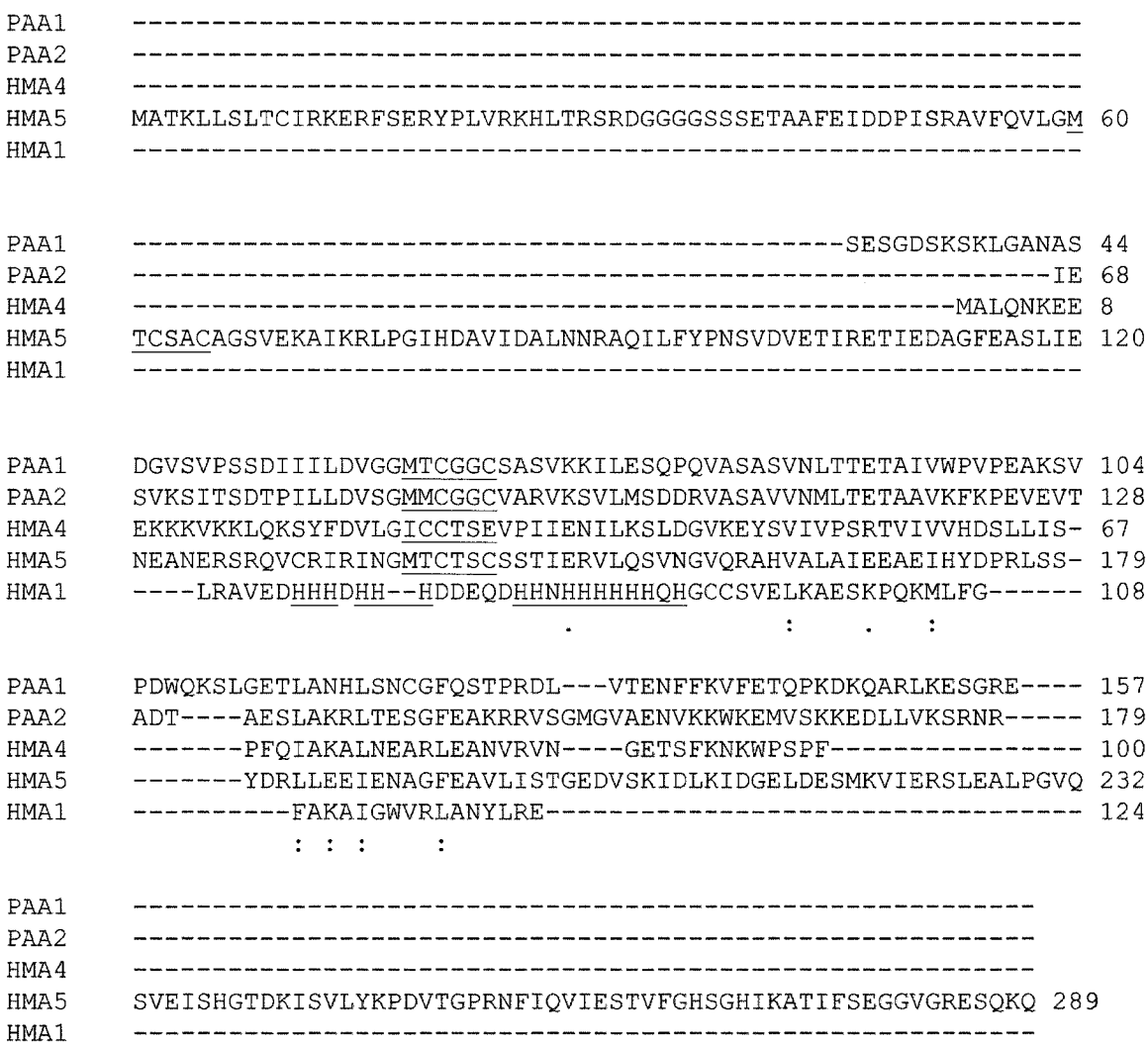

Figure 1. Sequences and alignment of Heavy Metal Associated (HMA) transporter encoded regions cloned into the yeast two-hybrid system. Underlined residues are putative metal binding sites, and dots symbolize residue similarity.

```
ATX1    ------------------------------------------------------------
CCH     ------------------------------------------------------------
CCP     ASARGSDVQIRRKSSADVSDLRRSRSSLLSSSSRYLLK-DHKSLKGDDKDLWLSSDRSKD 142
CCS     ATALTSDRNLHQEDRAMPQLLTEFMVDMTCEGCVNAVKNKLETIEGIEKVEVDLSNQVVR 127
CutA    ------------------------------------------------------------

ATX1    --------MLKDLFQAVSYQNTASLS-----LFQALSVVESKAMSQTVVLRVAMTCEGCV 47
CCH     ------------------------------------------MAQTVVLKVGMSCQGCV 17
CCP     LILYRDRNVTSSASSSSSSSSSFSS-----SVTNVSSPAPSTDDQVVVLRVSIHCKGCE 197
CCS     ILGSSPVKAMTQALEQTGRKARLIGQGVPQDFLVSAAVAEFKGPDIFGVVRFAQVSMELA 187
CutA    ----------------MEESSKTVPS-----IVVYVTVPNREAGKKLANSIVQEKLAACV 105
                                                          .

ATX1    GAVKRVLGKMEGVESFDVDIK---EQKVTVKG----------NVQPDAVLQTVTKTGKKT 94
CCH     GAVNRVLGKMEGVESFDIDIK---EQKVTVKG----------NVEPEAVFQTVSKTGKKT 64
CCP     GKVRKHISKMEGVTSYTIDLA---TKKVTVVG----------KITPVGLVESISKV-KFA 243
CCS     RIEANFTGLSPGTHSWCINEYGDLTNGAASTGSLYNPFQDQTGTEPLGDLGTLEADKNGE 247
CutA    NIVP---G-IESVYEWEGKVQSD-SEELLIIK-----------TRQSLLEPLTEHVNAN 152
               .   .. .:  .      :                    . . .:    :

ATX1    AFW--EAEGET--------------------------AKA-------------------- 106
CCH     SYWPVEAEAEPKAEADPKVETVTETKTEAETKTEAKVDAKADVEPKAAEAETKPSQV--- 121
CCP     QLWPSSSSPPFP-------------------------HIPNYSLLKS------------ 265
CCS     AFYSGKKEKLKVADLIGRAVVVYKTDDNKSGPGLTAAVIARSAGVGENYKKLCSCDGTVI 307
CutA    HEYE---------------------------------------------------------- 182
          :

ATX1    -------------
CCH     -------------
CCP     -------------
CCS     WEATNSDFVASKV 320
CutA    -------------
```

Figure 2. Sequences and alignment of copper chaperones and putative copper related proteins expressed in a yeast two-hybrid system. Underlined residues are putative metal binding sites, and dots symbolize residue similarity.

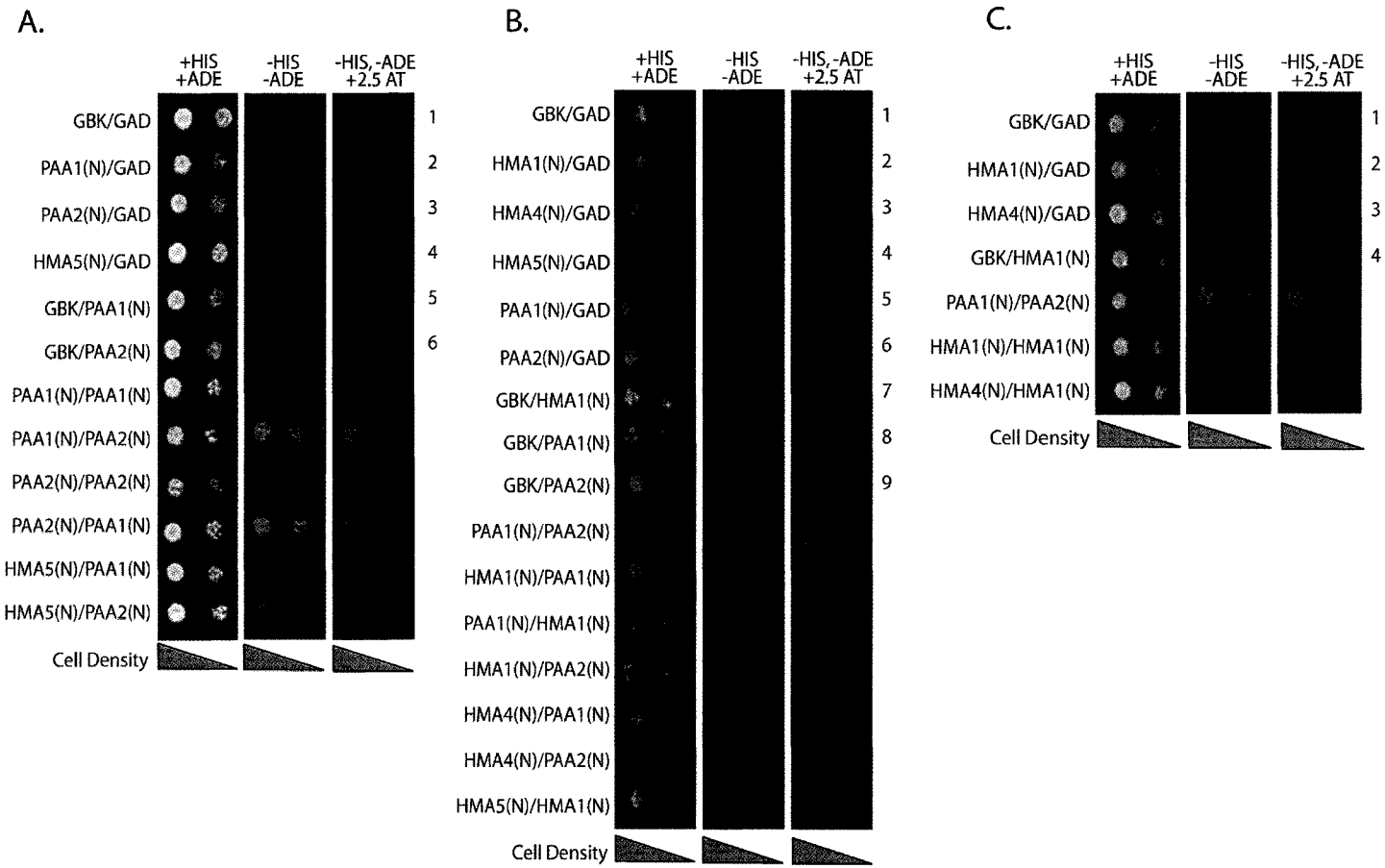

Figure 3. Interactions between HMA transporters of *Arabidopsis* as measured by yeast two-hybrid assays. Yeast two-hybrid spot assays of Heavy Metal Associated (HMA) Family transporter-transporter interactions showing interactions on plates lacking histidine and adenine supplemented with 2.5 mM 3-aminotriazol (AT). Panel A shows putative Cu transporter - Cu transporter interactions, while Panel B shows copper transporters with putative zinc transporters, and Panel C shows putative zinc transporter-transporter interactions. Numbered lanes indicate negative controls and the interaction between PAA1/PAA2 was used as a positive control after discovery.

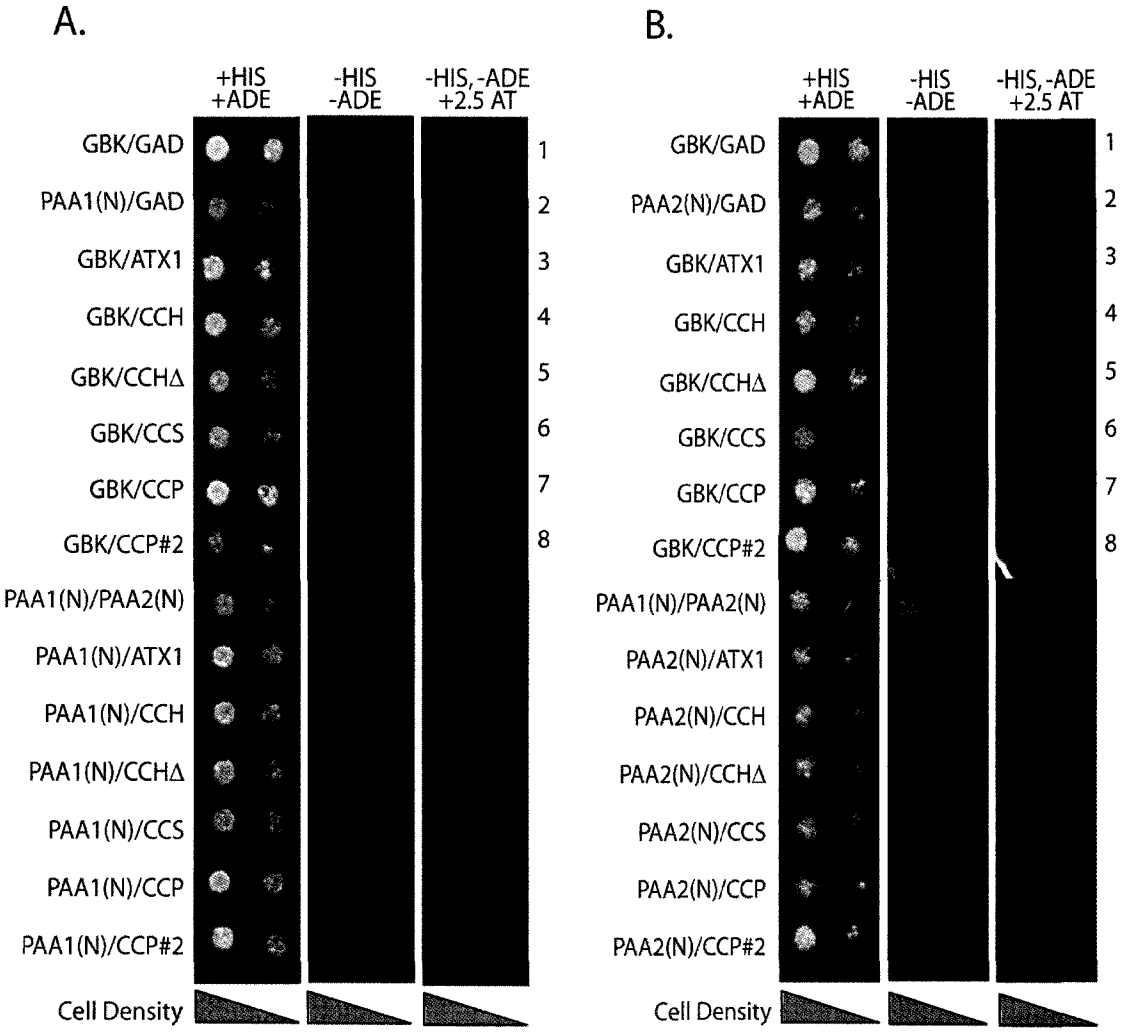

Figure 4. Interactions between PAA transporters and copper chaperones of *Arabidopsis* as measured by yeast two-hybrid assays. Yeast two-hybrid spot assays of PAA(N) transporters and copper chaperones showing interactions on plates lacking histidine and adenine supplemented with 2.5 mM 3-aminotriazol (AT). Numbered lanes indicate negative controls and the interaction between PAA1/PAA2 was used as a positive control.

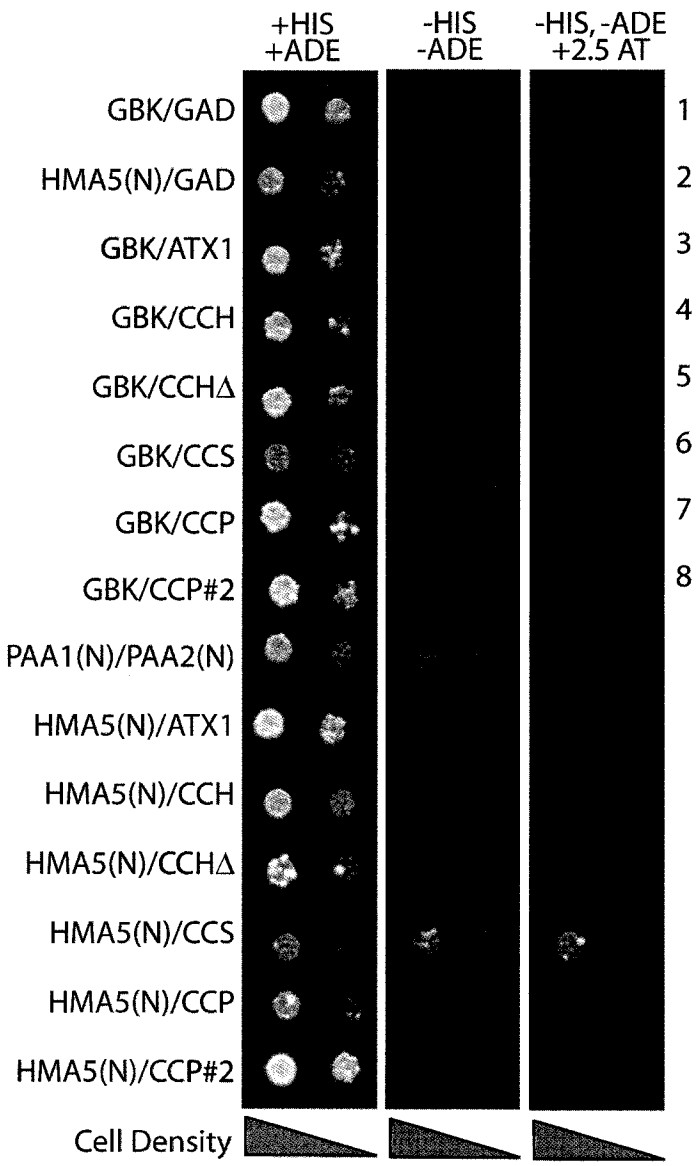

Figure 5. . Interactions between the HMA5 transporter and copper chaperones of *Arabidopsis* as measured by yeast two-hybrid assays. Yeast two-hybrid spot assay of HMA5(N) transporter and copper chaperones showing interactions on plates lacking histidine and adenine supplemented with 2.5 mM 3-aminotriazol (AT). Numbered lanes indicate negative controls and the interaction between PAA1/PAA2 was used as a positive control.

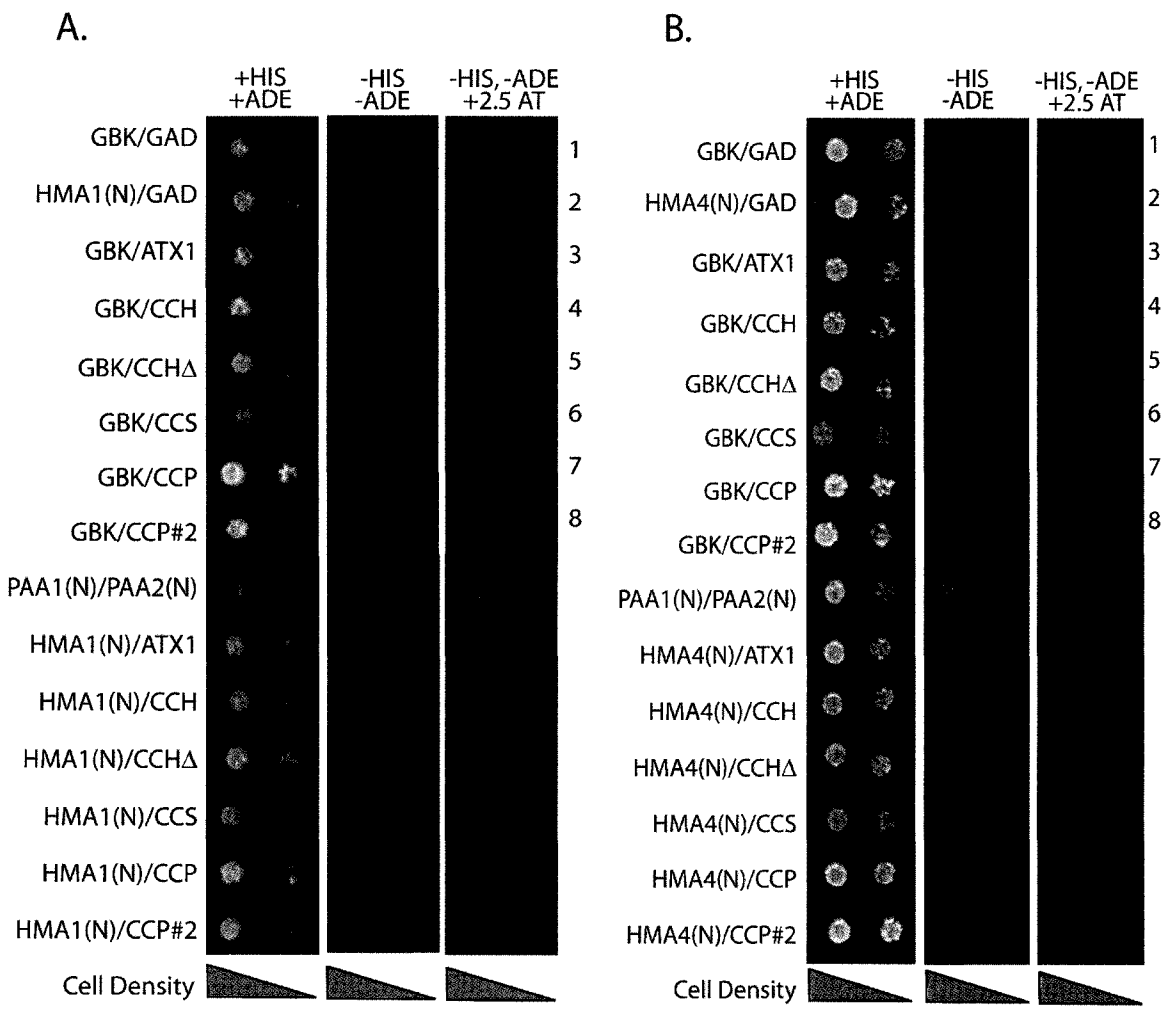

Figure 6. . Interactions between HMA transporters and copper chaperones of *Arabidopsis* as measured by yeast two-hybrid assays. Yeast two-hybrid spot assay of putative zinc transporters, HMA1(N) and HMA4(N), with copper chaperones (Panels A and B, respectively) showing interactions on plates lacking histidine and adenine supplemented with 2.5 mM 3-aminotriazol (AT). Numbered lanes indicate negative controls and the interaction between PAA1/PAA2 was used as a positive control.

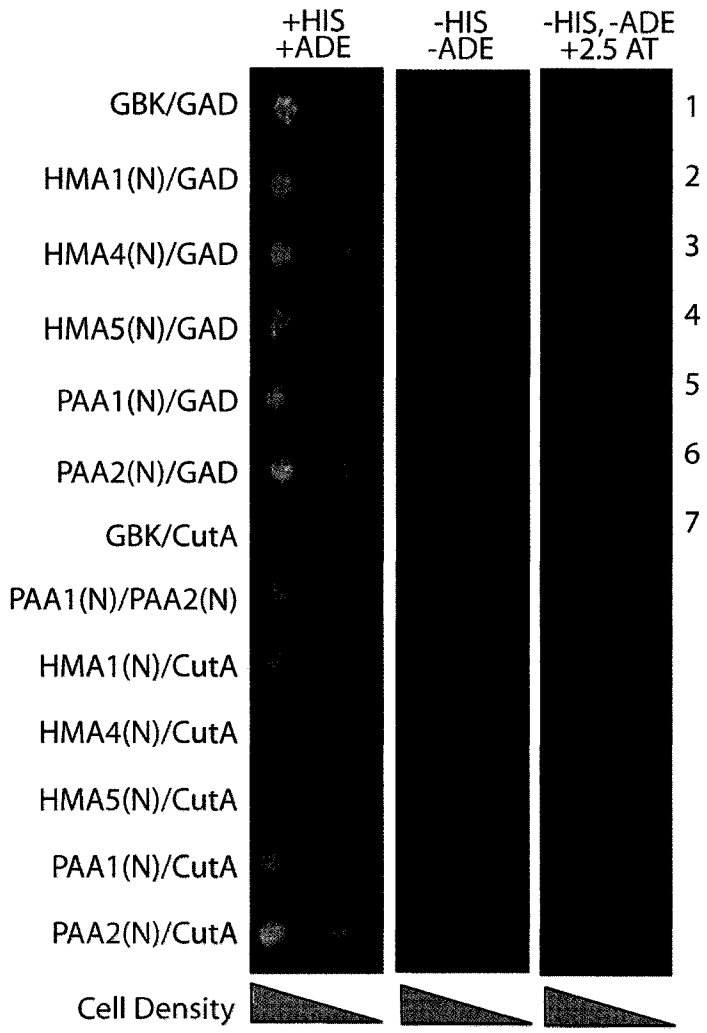

Figure 7. . Interactions between HMA transporters and the CutA protein of *Arabidopsis* as measured by yeast two-hybrid assays. Yeast two-hybrid spot assay of putative copper chaperone CutA and amino termini of Heavy Metal Associated transporters showing interactions on plates lacking histidine and adenine supplemented with 2.5 mM 3-aminotriazol (AT). Numbered lanes indicate negative controls and the interaction between PAA1/PAA2 was used as a positive control.

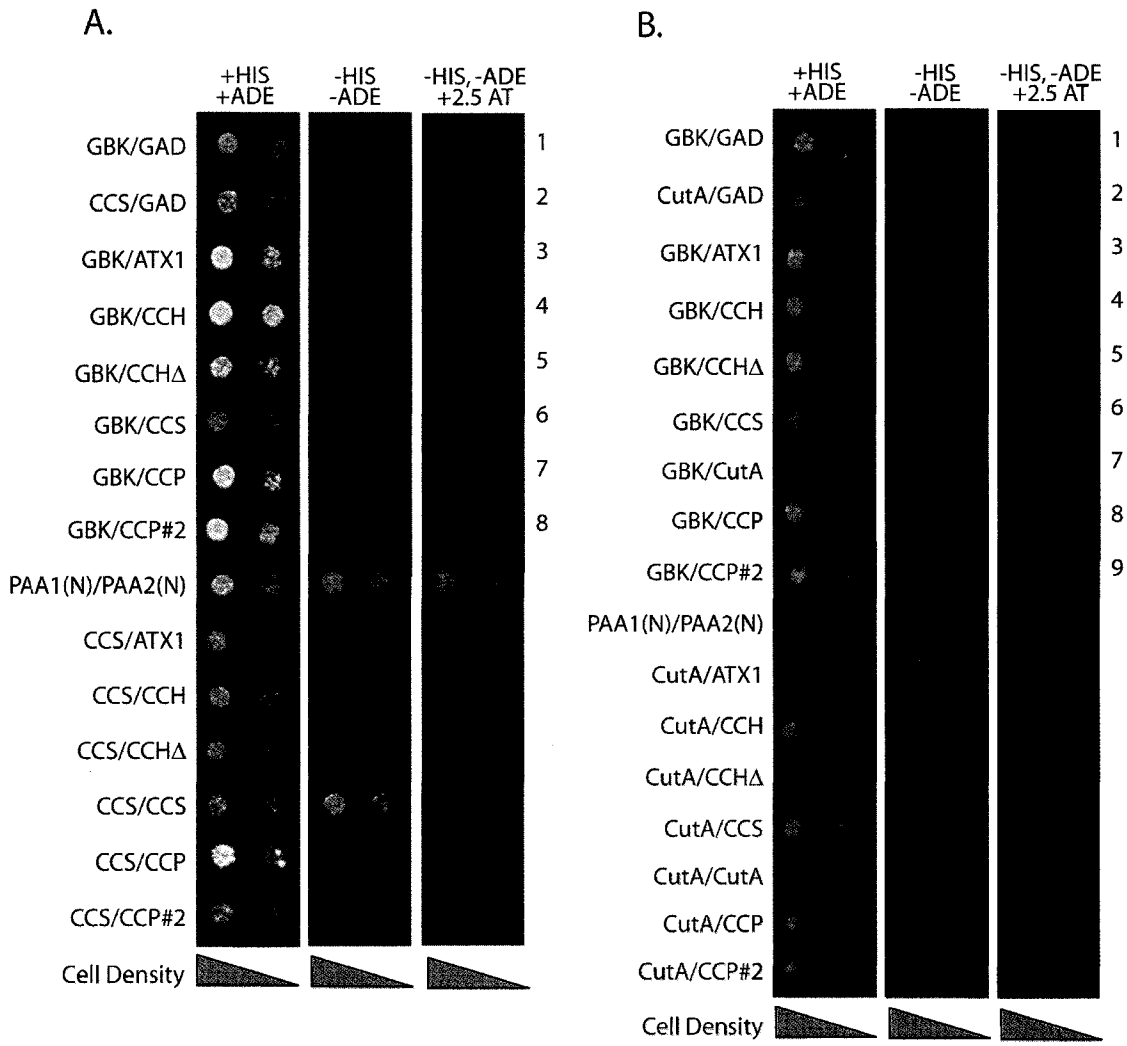

Figure 8. Yeast two-hybrid spot assays of putative copper chaperones, CCS and CutA, with copper chaperones (Panels A and B, respectively) showing interactions on plates lacking histidine and adenine supplemented with 2.5 mM 3-aminotriazol (AT). Numbered lanes indicate negative controls and the interaction between PAA1/PAA2 was used as a positive control.

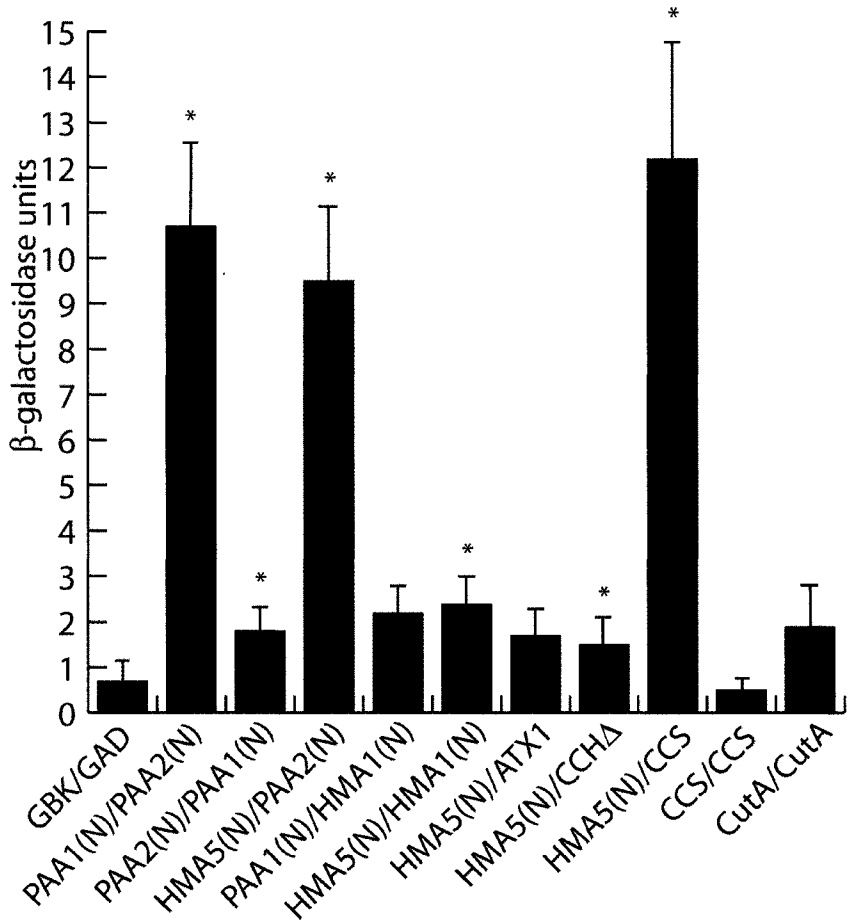

Figure 9. A quantitative estimate of copper protein interactions from yeast two-hybrid using β-galactosidase activity liquid assays. Average values displayed were calculated as Miller units and can be defined as the activity which hydrolyzes 1 μmol of chlorophenol red-β-D-galactopyranoside (CPRG) to chlorophenol red and D-galactose per minute per cell [20,21]. Significant difference between positive interactions and empty vector control is indicated (Student's *t* test, $p<0.05$).

	HMA1(N)	PAA1(N)	PAA2(N)	ATX1	CCH	CCH Δ	CCS	CutA	CCP	CCP#2	**pGAD**
HMA1(N)	-	-	-	-	-	-	-	-	-	-	
HMA4(N)	-	-	-	-	-	-	-	-	-	-	
HMA5(N)	+	-	**+**	+	-	+	**+**	-	-	-	
PAA1(N)	+	-	**+**	-	-	-	-	-	-	-	
PAA2(N)		+	-	-	-	-	-	-	-	-	
CCS			-	-	-	-	+	-	-	-	
CutA			-	-	-	-	-	+	-	-	
pGBK											

Figure 10. Summary of yeast two-hybrid spot assay results. The symbol + indicates growth on plates lacking histidine and adenine with large, bold symbols indicating a very strong interaction, while the symbol - indicates no growth on the same plates. Diagonal lines denote that no interaction was tested.

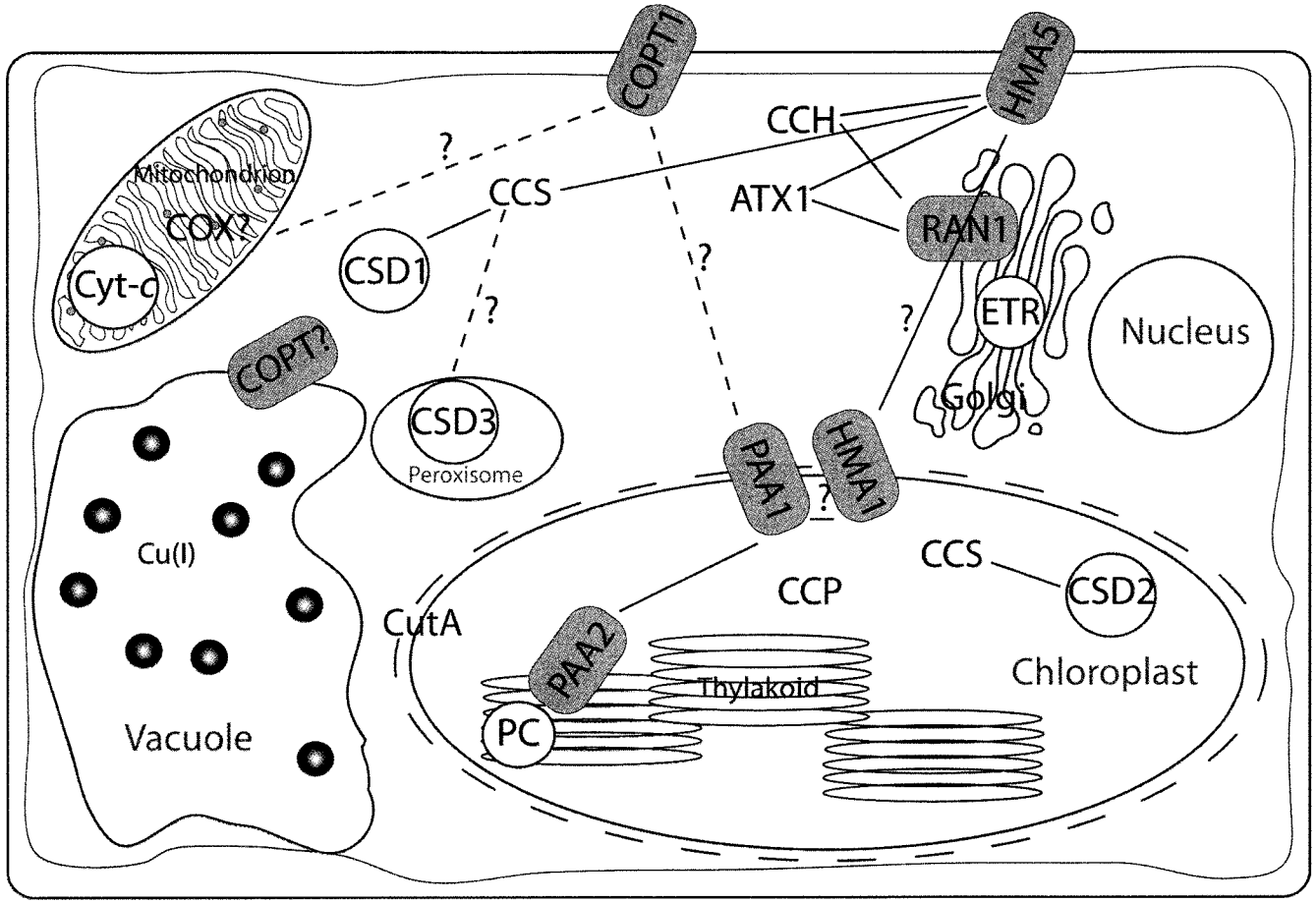

Figure 11. A revised illustration of copper transport pathways in *Arabidopsis thaliana* with new possible routes discovered using the yeast two-hybrid technique (new interactions are shown in red, solid lines depict interactions demonstrated through published experimental data, dashed lines are hypothetical pathways, gray rectangles are copper transporters, and white circles represent copper targets).

CHAPTER 7

Conclusions

The three most abundant trace elements in biochemical systems are iron, zinc, and copper [1]. Copper is an essential micronutrient that is required for several biological processes in *Arabidopsis thaliana*. Enzymes that contain copper ions have three major functions: dioxygen transport, catalytic, or copper transport/sequestration [1]. Plastocyanin is a small (10 kDa) protein that is located inside the lumen of plant chloroplasts as well as some cyanobacteria and green algae. Some cyanobacteria and Chlamydomonas can use the iron containing cytochrome c_6 protein for transport of electrons in photosynthesis instead of plastocyanin [2]. However, higher plants such as *Arabidopsis* do not have an alternative and require plastocyanin for photosynthetic activity.

The chloroplast is a complex organelle that must communicate with the rest of the cell to acquire copper for photosynthesis. Since plastocyanin is one of the most abundant proteins in photosynthetic tissue the demand for copper within the organelle is extremely high. Several P_{1B}-type ATPase transporters and metallochaperones have previously been identified in *Arabidopsis* and

contribute to copper ion delivery within the organism. This research focused on the proteins that are involved in transport of copper ions with the aim of determining their placement within the delivery pathway. As a result a better understanding of cellular copper homeostasis was achieved and several new possible pathways were identified.

The yeast two-hybrid technique was utilized to screen 65 different protein-protein interactions in *Arabidopsis*. A total of ten positive interactions were identified, however two seem the most plausible within a plant cell (PAA1(N)/PAA2(N) and HMA5(N)/CCS). An interaction between the amino terminal domains of PAA1 and PAA2 was described which led to a more indepth look at these transporters. Both *paa1* and *paa2* mutants have previously been described [3,4] and phenotypes include, decreased growth rate, a decrease in photosystem II efficiency, as well as a decrease in accumulation of plastocyanin. We investigated these mutants further and noted differences in chloroplast structure compared to wildtype that may be a result of stress-related damage. Protein levels of PC, CSD1, CSD2, FeSOD, and CCS were all affected by the mutation, especially in low copper treatments. When root and shoot tissue was analyzed individually we observed differential regulation of CSD1 and CSD2 in different organs. Similar to whole plant tissue PC, SOD, and CCS were affected differently in the mutant plant lines. With the data we obtained, we propose that the ATX-like domains of PAA1 and PAA2 directly interact with one another to pass copper ions for the delivery of the metal to plastocyanin in the thylakoid membrane.

The *Arabidopsis CCS* gene (At1g12520) contains six exons with two separate ATG sites located in the first exon. We provide experimental data that suggests that *AtCCS* encodes a functional homologue to yeast Ccs1p/Lys7p, a copper chaperone for SOD. Through GFP fusion analysis we have localized this protein to the chloroplast where it may supply copper to the stromal Cu/ZnSOD. Furthermore, *AtCCS* mRNA expression levels are up-regulated in response to Cu-feeding and senescence. In addition to plastocyanin, the chloroplast contains two different isoforms of superoxide dismutases (FeSOD and CSD2). We observed a switching between a Cu-enzyme (CSD2) and an Fe-enzyme (FeSOD) in chloroplasts in response to cofactor bioavailability. We propose that in low copper conditions metal ions are directed towards plastocyanin in the chloroplast and FeSOD is the active SOD enzyme. However, under sufficient copper concentrations some copper ions can be diverted to CSD2 in the stroma.

New experimental data was then published that localized the CCS protein to both the cytosol as well as the stroma [5]. We obtained a CCS knockout mutant plant line and produced two separate CCS overexpressing lines to further investigate this protein and better understand its role in copper homeostasis. We have reported that the alteration of CCS protein levels slightly affect the expression of the target SOD proteins. We observed a growth phenotype based on primary root length and a chlorophyll fluorescence phenotype that is associated with CCS overexpression. There is evidence that CCS is regulated by copper, especially at low concentrations. Data from a developmental study indicates that CCS is expressed differently in varying

tissue types. This data combined with the yeast two-hybrid analysis suggests that the CCS protein can possibly aid in cellular detoxification or long distant transport of copper through different tissue types.

Recently, a third P_{1B}-type ATPase, HMA1, has been localized to the chloroplast [6] and some data has suggested that HMA1 could be involved in both copper and zinc transport across the envelope. It was proposed that HMA1 could be an alternative copper transporter in the envelope which translocates the ion for CSD2 activity. We obtained *hma1* mutant seeds from Norbert Rolland (Université Joseph Fourier, Grenoble, France) and compared them to *paa1* and *paa2* mutant plant lines. If all three transporters are located in the chloroplast and translocate copper ions, we might expect that mutations in the genes create similar biochemical and physiological effects on plants. We report that the *hma1*, *paa1*, and *paa2* plant lines have a decreased photosystem II efficiency compared to wildtype plants; however, copper feeding does not affect *hma1* plants. We note distinct differences between the chloroplast structure of *paa1* and *hma1* mutant plants. Finally, we observe no differences in proteins expression levels of PC, FeSOD, CSD1, CSD2, and CCS between *hma1* and control plants. This data suggests that HMA1 does not transport copper for CSD2 activity and we propose that the transporter functions in the translocation of an ion other than Cu(I) across the chloroplast envelope that affects photosynthetic activity.

We believe that the research presented here has made a significant contribution to the understanding of copper homeostasis in *Arabidopsis* plant cells. We have provided experimental data that shows a direct interaction

between two separate P-type ATPase transporters. Prior to this investigation an interaction of this type has not been described. We have identified two novel pathways for copper delivery within the cell between PAA1 and PAA2 as well as between CCS and HMA5. We have determined that the superoxide dismutase enzymes and CCS expression levels are regulated by the availability of copper within the cell. Additionally, we describe physiological responses that occur in *Arabidopsis* that are a result of metal supply, tissue demands, or altered protein expression. We believe that the research conducted here on copper transport proteins can be combined with the genetic and genomic analysis that has been done to provide a more broad understanding of metal homeostasis in higher plant systems.

References

[1] Bhattacharya, P.K. (2005) Metal ions in biochemistry, Alpha Science International Ltd. Harrow, U.K.

[2] Zhang, L., McSpadden, B., Pakrasi, H.B. and Whitmarsh, J. (1992). Copper-mediated regulation of cytochrome c553 and plastocyanin in the cyanobacterium Synechocystis 6803. J Biol Chem 267, 19054-9.

[3] Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K.K. and Pilon, M. (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15, 1333-46.

[4] Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M. and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17, 1233-51.

[5] Chu, C.C., Lee, W.C., Guo, W.Y., Pan, S.M., Chen, L.J., Li, H.M. and Jinn, T.L. (2005). A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. Plant Physiol 139, 425-36.

[6] Seigneurin-Berny, D., Gravot, A., Auroy, P., Mazard, C., Kraut, A., Finazzi, G., Grunwald, D., Rappaport, F., Vavasseur, A., Joyard, J., Richaud, P., and Rolland, N. (2006). HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem 281, 2882-92.

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