Cell Biology of Copper

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Abstract The transition metal, copper (Cu), is an essential micronutrient for normal plant growth and development. Copper is a cofactor of proteins involved in photosynthesis, respiration, ethylene perception, removal of superoxide radicals, and cell wall modification. The biochemical reactions catalyzed by most Cu enzymes in plants are known. However, in many cases we are not yet sure about the biological function of these Cu proteins. Copper delivery to Cu proteins has evolved with a set of evolutionarily conserved transporters and metallo-chaperones. Analysis of Cu transporter and metallo-chaperone loss of function mutants has increased our understanding of the localization and biological function of many Cu delivery mechanisms and target Cu proteins. Studies examining the regulation of Cu transporters, metallo-chaperones, and Cu proteins have revealed an elegant system to regulate Cu homeostasis. Copper in excess is toxic while Cu deficiency can lead to decreased photosynthetic activity and reproductive success. To avoid Cu deficiency or toxicity symptoms in a sub-optimal environment, plants are capable of directing Cu delivery based on their needs via regulation of Cu proteins and delivery systems. For many Cu proteins, a network of Cu microRNAs, under the control of a SPL7 transcription factor, orchestrates the prioritization of Cu delivery based on Cu availability.

1 Introduction

Copper (Cu) is an essential micronutrient for life, and it is important for many cellular processes in numerous organelles and compartments. In cells, Cu is found in two common states, Cu(I) (reduced) or Cu(II) (oxidized). Cu ions often act as

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cofactors in Cu-proteins that are associated with electron transfer reactions and redox reactions involving oxygen (Linder and Goode 1991). However, the redox-active properties of Cu could also cause unwanted and uncontrolled reactions if it is left alone as a free ion in the cell. Free Cu ions could lead to the formation of toxic hydroxyl radicals, which are capable of damaging macromolecules (Halliwell and Gutteridge 1984). An extensive network of Cu transporters and metallo-chaperones has therefore evolved to bind and shuttle Cu in a manner that ensures proper Cu delivery to Cu proteins in various cellular organelles and compartments.

2 Functions of Cu Proteins in Plants

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

2.1 Plastocyanin

The plastocyanin protein was first discovered in Algae (Katoh 1960). Plant (Poplar) plastocyanin was one of the first Cu proteins with a known crystal structure, which showed that the protein binds its Cu via a cysteine, a methionine and two histidine ligands (Colman et al. 1978). Plastocyanin is the most abundant protein in the thylakoid lumen where it functions as a mobile carrier of electrons from the cytochrome-b6/f complex to PSI (Kieselbach et al. 1998; Schubert et al. 2002). Therefore, it has a critical role in both linear and cyclic electron flows (Raven et al. 1999). A higher plant (*Silene pratensis*) plastocyanin cDNA sequence was first published in 1986 (Smeekens et al. 1986). *Arabidopis* has two plastocyanin genes (Vorst et al. 1993; Kieselbach et al. 2000) that encode proteins with highly similar secondary structures and seemingly redundant functions (Pesaresi et al. 2008). Unlike some cyanobacteria and green algae, plastocyanin is the only protein that can accept electrons from the cytochrome-b6/f complex in higher plants (Molina-Heredia et al. 2003), and *Arabidopsis* mutants with insertions in both plastocyanin genes are seedling-lethal on soil (Weigel et al. 2003). Arguably, plastocyanin is the most important Cu protein in photosynthetically growing plants.

2.2 Cytochrome c Oxidase

In mitochondria, Cu is required for the function of cytochrome *c* oxidase (COX), the proton-pumping terminal oxidase in the inner membrane (Carr and Winge 2003). This multi-subunit protein contains three Cu ions as cofactors in two Cu

sites, in addition to heme. At least ten subunits make up the cytochrome *c* oxidase of higher plants. In yeast, three conserved mitochondrial encoded subunits (COX1, 2, and 3) form the core of the enzyme and contain the Cu binding sites (Carr and Winge 2003). Coordinated expression of nuclear and mitochondrial encoded COX genes is required for cytochrome *c* oxidase activity. Nuclear encoded COX subunit genes may help control tissue specific activity of this enzyme (Welchen et al. 2004). It is well established that a lower cytochrome *c* oxidase activity is one effect of severe Cu deficiency in plants (Marschner 1995). Along with COX, plants have the Fe-containing alternative oxidase (AOX). This protein accepts electrons from ubiquinone and reduces O_2 to water without proton pumping. AOX activity may prevent over-reduction of the quinone pool in cases where downstream electron transport is less efficient as a result of a stressful environment (Clifton et al. 2006).

2.3 Cu/Zn Superoxide Dismutase

Together with Zn, Cu is also a cofactor of Cu/Zn superoxide dismutase (Cu/ZnSOD) proteins that function in reactive oxygen species metabolism (Bowler et al. 1992). SOD enzymes catalyze the conversion of two superoxide ions and two protons to peroxide and molecular oxygen. Three genes encode for Cu/ZnSOD in the *Arabidopsis* genome: CSD1 is active in the cytosol, CSD2 in the stroma, and CSD3 in peroxisome (Kliebenstein et al. 1998). Homologs of these Cu/ZnSOD genes have been found in the genomes of other plant species, but they are not found in *Chlamydomonas*.

2.4 Ethylene Receptors

The ethylene receptors are Cu-binding proteins (Rodriguez et al. 1999) that are active in an early endomembrane system compartment, most likely the endoplasmic reticulum (Chen et al. 2002). For proper ethylene perception and responses, Cu must be delivered to the lumen of the endomembrane system (Hirayama et al. 1999).

2.5 Phytocyanins

The phytocyanins constitute a plant specific blue Cu protein family. Phytocyanins are structurally similar to plastocyanin and bind a single Cu per polypeptide. Phytocyanins include plantacyanin, stellacyanin, and uclacyanin (Nersissian et al. 1998). These proteins differ from plastocyanin in that their precursors harbor signal

peptides that direct them into the endoplasmic reticulum and secretory pathway. The biological role of these phytocyanins is not fully clear. It has been suggested that some may mediate lignin polymerization (Nersissian et al. 1998). In Lilly, plantacyanin functions as a signaling molecule in the transmitting tract of the pistil (Kim et al. 2003), and evidence in *Arabidopsis* also suggests a role in reproduction (Dong et al. 2005).

2.6 Laccase and Ascorbate Oxidase

Multi-copper oxidases (MCO) form a super-family of proteins that bind four Cu ions (Nakamura and Go 2005) and include the ferroxidases (in yeast and *Chlamydomonas*), ceruloplasmin (in mammals), ascorbate oxidase, and laccases (in plants and fungi). In plants, apoplastic ascorbate oxidases were shown to have roles in cell expansion, plant biomass production, and salt tolerance (Pignocchi et al. 2003; Yamamoto et al. 2005). Laccase catalyzes the oxidation of a suitable substrate molecule (phenols and aromatic or aliphatic amines) to the corresponding reactive radicals with the production of water and oligomers (Gavnholt and Larsen 2002). In plants, laccases are apoplastic and encoded by a multi-gene family with 17 genes in *Arabidopsis* (McCaig et al. 2005; Cai et al. 2006). Different laccases are expressed in different organs and at different developmental stages of growth in *Arabidopsis* (McCaig et al. 2005). Insertion mutants for most laccases do not show a phenotype except for Lac2 (altered root elongation during de-hydration), Lac8 (early flowering), and Lac 15 (altered seed color) (Cai et al. 2006). The mutation in Transparent Testa-10 (TT10), an *Arabidopsis* mutant with a lack in seed color, was mapped to Lac15 which is expressed in the developing seed and functions in the formation of proanthocyanidin or tannin (Pourcel et al. 2005). Another possible role of laccase is in Fe acquisition (Hoopes and Dean 2004). All other proposed biological functions of laccases in plants can be summarized as "cell wall modeling"; this includes roles in lignin synthesis, maintenance of cell wall structure and integrity, response to stress, and wound healing (Sterjiades et al. 1992; Bao et al. 1993; Dean and Eriksson 1994; Ranocha et al. 2002; Liang et al. 2006).

2.7 Polyphenol Oxidase

Polyphenol oxidase (PPO) or tyrosinase was the first discovered Cu enzyme in plant plastids (Arnon 1949). It is found in the thylakoid lumen and contains a dinuclear Cu center. PPO catalyzes the conversion of monophenols to ortho-diphenols and ortho-dihydroxyphenols to ortho-quinones, resulting in black or brown pigment deposits (for review see Mayer 2006). In tomato, the PPOs are encoded by a gene family with seven members (Newman et al. 1993) that are differentially expressed (Thipyapong et al. 1997). Wounding, stress, pathogen, and herbivore attack have

been shown to induce PPO activity in different plant species, suggesting a role for PPO in plant resistance to stress and pathogens (Mayer 2006). PPO is not ubiquitous and there is no homolog for PPO in *Arabidopsis* (Schubert et al. 2002).

2.8 Amine Oxidase

Amine oxidases contain a single Cu as well as a special topa quinone cofactor that is formed by post-translational modification of a conserved tyrosine residue (Kumar et al. 1996). These secreted enzymes catalyze the oxidative deamination of primary amines to aldehydes in a reaction that requires free radicals and also produces hydrogen-peroxide (Frebort et al. 2000). Poly-amines such as spermine are likely substrates for these enzymes. The Cu atom is bound by three histidine residues and is required not only for the post-translational formation of the topa quinone cofactor but also for the regular catalytic cycle (Kumar et al. 1996). Amine oxidases are reported to be the most abundant Cu proteins in the apoplastic space of peas. Proposed functions include roles in cell wall differentiation, which in turn could be significant for stomatal closure (An et al. 2008), wound healing (Rea et al. 2002; Angelini et al. 2008), and responses to pathogen attack (Rea et al. 2002; Marina et al. 2008). The observed amine oxidase expression pattern and timing in tobacco would be consistent with a role in either peroxide dependent protein cross-linking or lignification (Paschalidis and Roubelakis-Angelakis 2005).

2.9 Other Roles of Cu in Plants

While determining the structure of CNX1, an enzyme that functions in molybdenum cofactor synthesis, it was found that a Cu ion temporarily occupies the site for molybdenum insertion in the bound molybdopterin substrate (Kuper et al. 2004). This observation now links Cu metabolism to nitrogen assimilation and phytohormone biosynthesis, which are functions of molybdenum cofactor requiring enzymes. Cu may also play a role in thylakoid grana stacking (Bernal et al. 2006). Unlike what has been found for yeast and *Chlamydomonas*, there is no direct requirement for Cu in Fe acquisition.

3 Cu Movement in and out of Root Cells

3.1 Cu Uptake

In root cells, Cu enters the cytosol by a cell membrane COPT-family transporter (Kampfenkel et al. 1995). The family of COPT transporters belongs to a highly conserved Ctr-like Cu transporter family also found in yeast and humans

(Dancis et al. 1994). COPT transporters have three transmembrane domains, a likely *N*-terminal metal binding domain, and an essential MXXXM transmembrane domain (Puig et al. 2002). *Arabidopsis* encodes five COPT transporters (COPT1 – 5). Of these, four are expressed and these proteins likely import Cu into the cytosol, though their sub-cellular location is not determined. Information on COPT1 promoter fusion and antisense lines suggest that COPT1 is involved in Cu uptake from the surrounding growth medium at root tips (Sancenon et al. 2004). COPT1 antisense lines exhibited elongated root growth when compared to wild-type plants, and the phenotype could be partially restored upon Cu feeding in the medium (Sancenon et al. 2004). COPT2 is also likely involved in cellular uptake considering its expression in root and leaf tissues, along with up-regulation of transcripts during limited Cu growth, similarly seen for COPT1 (Sancenon et al. 2003). COPT3 and COPT5 are highly expressed in aerial tissues (Sancenon et al. 2003), and may serve to transport Cu from intracellular stores.

Ctr-like proteins transport Cu in its reduced form (Eisses and Kaplan 2005), but most extracellular Cu in soil is oxidized as Cu(II). *Arabidopsis* and dicot species utilize root surface ferric reductases, such as FRO2, for uptake of Fe in its reduced form (Robinson et al. 1999). It is also possible that ferric reductases could reduce Cu for import (Welch et al. 1993). When plants are fed an excess of Cu, Fe concentrations decrease; the opposite is also true during limited Cu growth (Welch et al. 1993; Chen et al. 2004). Interestingly, FRO3, localized in roots and vasculature, exhibits increased expression during Cu deficient growth (Mukherjee et al. 2006). However, FRO activity has not been reported to reduce Cu(II). In addition to COPT transporters, ZIP2 and ZIP4 (ZIP family transporters) have been reported to complement the yeast *ctr1* mutant, that is deficient in Cu uptake (Wintz et al. 2003). ZIP2 transcript expression is highest in root tissue while ZIP4 expression is high in both root and leaf tissue, and they respond to Cu status (Wintz et al. 2003).

3.2 Cu Export and Intercellular Reallocation

The HMA5 (Heavy Metal Associated 5) Cu transporter likely supplies Cu to apoplastic Cu oxidases and laccases (see below). It also plays an important role in removing excess Cu from the cytosol of root tissues (Andrés-Colas et al. 2006; Kobayashi et al. 2008). Root tissues in *hma5* loss of function mutants accumulate elevated levels of Cu when compared to wild-type plants, and *hma5* mutants are more sensitive to Cu feeding (Andrés-Colas et al. 2006). Delivery of Cu within the cytosol of plants to RAN1 (HMA7, see below) (Hirayama et al. 1999) and HMA5 may be accomplished by two homologs of the yeast Atx1 Cu chaperone, ATX1 (Andrés-Colas et al. 2006; Puig et al. 2007) and CCH (Himelblau et al. 1998). Both ATX1 and CCH from *Arabidopsis* are able to complement the yeast *atx1* mutant, and they interact with the *N*-terminal domain of *Arabidopsis* HMA5 and RAN1 (ATX1 only) in a yeast two hybrid system (Andrés-Colas et al. 2006; Puig et al. 2007). The yeast

Atx1 and ATX1 in *Arabidopsis* are similar, however, CCH contains an added plant specific *C*-terminal extension (Mira et al. 2001a; Puig et al. 2007). Interestingly, this *C*-terminal addition negatively affects interactions with HMA5, but a positive interaction was observed when the *C*-terminal region of CCH was removed (Andrés-Colas et al. 2006; Puig et al. 2007). CCH has been found in phloem-endonucleated cells, and it is possible that the additional *C*-terminal region allows for symplastic intercellular Cu trafficking through plasmodesmata (Mira et al. 2001b; Andrés-Colas et al. 2006). Up-regulation of ATX1 and CCH has been reported for plants undergoing Cu deficiency, senescence, mechanical and oxidative stress, along with jasmonic acid treatments in *Arabidopsis* (Himelblau et al. 1998; Mira et al. 2001b; Puig et al. 2007), and in poplar (Lee et al. 2005).

3.3 Root to Shoot Cu Translocation

Since HMA5 is involved with Cu movement from the symplast to apoplast, and is highly expressed in roots, it is possible that HMA5 is also involved in transporting Cu into the xylem. If so, it is not the only mechanism to load Cu into the xylem considering that *hma5* loss of function mutants were able to maintain much of the Cu translocation to shoot tissues (Andrés-Colas et al. 2006). No other mechanism for Cu loading into the xylem has been suggested or identified. Once in the xylem, long distance Cu translocation to aerial tissues may involve the chelator nicotianamine. As a methionine-derived compound, nicotianamine chelation of Fe in xylem sap for translocation has been suggested (for review see Briat et al. 2007). Nicotianamine has also been shown to have a high affinity for Cu binding in tomato xylem sap, and less than 0.5% of total xylem Cu was found as free Cu(II) ions (Liao et al. 2000). This suggests that Cu in xylem sap is mostly chelated. The tomato mutant *chloronerva*, which lacks nicotianamine, also supports the idea that nicotianamine is involved with long distance transport of heavy metals. *chloronerva* mutant plants exhibit increased Cu concentrations in root tissues and decreased xylem and shoot levels compared to wild-type (Pich and Scholz 1996). Upon application of nicotianamine to these mutants it was observed that root Cu concentrations decreased while xylem and shoot levels increased, especially in young leaves (Pich and Scholz 1996). In addition, tobacco plants over-expressing a nicotianamine aminotransferase (NAAT) gene, which creates a nicotianamine shortage in tobacco, led to Cu deficiencies in leaves and problems associated with reproduction (Takahashi et al. 2003).

3.4 Excess Cu

In some cases plant cells may have to deal with excessive Cu. Plants such as *Arabidopsis* do not accumulate high levels of Cu in tissues and are often sensitive to elevated Cu. During sub-toxic Cu excess, plants may be able to chelate Cu using

a cysteine-rich metallothionein (MT). *Arabidopsis* contains several MT genes, some of which are up-regulated during Cu excess (Zhou and Goldsbrough 1994; Guo et al. 2003). Another possible Cu chelator is phytochelatins, which are derived from glutathione (for review see Cobbett and Goldsbrough 2002). When plants lack both MT1a/MT2b and phytochelatin they exhibit a more severe phenotype on elevated Cu than MT or phytochelatin mutants alone (Guo et al. 2008). Simply moving Cu out of the cell may also help maintain normal cellular Cu levels. Considering that *hma5* mutants are sensitive to Cu feeding, HMA5 is likely involved in detoxifying cells of excess Cu by moving the ions into extracellular spaces (Andrés-Colas et al. 2006; Kobayashi et al. 2008). HMA5, COPT1, and COPT2 transporters are regulated by Cu differently. HMA5 increases during Cu excess (Andrés-Colas et al. 2006) while the Cu importers COPT1 and COPT2 decrease (Sancenon et al. 2003, 2004), consistent with the role of HMA5 in preventing excess ions in the cell while also avoiding Cu toxic conditions for neighboring cells.

4 Intracellular Cu Delivery to Cu Protein Targets

4.1 Chloroplast: Cu Import into the Chloroplast

Import of Cu into stroma and thylakoid lumen is the most understood of any organelle in plants. The inner envelope membrane contains a metal-transporting P-type ATPase for *Arabidopsis*, PAA1 (HMA6), and is responsible for Cu import into the stroma (Tabata et al. 1997; Shikanai et al. 2003); while PAA2 (HMA8) imports Cu from the stroma into the thylakoid lumen (Abdel-Ghany et al. 2005; Bernal et al. 2007). Both are P_{1B} type pumps and members of the Heavy Metal Associated (HMA) transporter family (Axelsen and Palmgren 2001; Baxter et al. 2003). There are eight members in the HMA family. HMA1 to 4 are classified as possible Zn, Cd, Co, and Pb transporters; while HMA5 to 8 are classified as Cu and Ag transporters (Arguello 2003; Baxter et al. 2003). Both PAA1 and PAA2 Cu transporters have sub-cellular targeting information in the *N*-terminal region of the peptide, but the mechanism of protein import is not yet identified (Abdel-Ghany et al. 2005). PAA1 and PAA2 Cu transporters have eight predicted transmembrane domains with a heavy metal binding motif in the *N*-terminal region. In addition, they contain ATP binding, phosphatase, phosphorylation, and transmembrane CPC (amino acid) ion transduction domains (Mandal et al. 2004; Abdel-Ghany et al. 2005). Upon Cu metal binding and phosphorylation of P_{1B} type transporters, the Cu ion is transported across the membrane through changes in protein conformation (Arguello et al. 2007; González-Guerrero and Argüello 2008). Transport of heavy metals in most of these P_{1B} type transporters is thought to initiate in the sub-cellular compartment containing the heavy metal binding *N*-terminal region (Arguello et al. 2007; González-Guerrero and Argüello 2008). This would place the *N*-terminal

domains for PAA1 and PAA2 in the chloroplast envelope intermembrane space and stroma respectively. However, the orientation and mechanism for accepting and donating Cu is not yet known for PAA1 and PAA2.

Plastocyanin import into chloroplasts and thylakoids is conducted using the Tic/Toc and SecA/SecY-mediated pathways (for review see Schnell 1998), which translocate proteins in an unfolded state. Upon import, plastocyanin acquires its Cu cofactor for final assembly and stability. Mutants with impaired Cu transport (*paa1* and *paa2* loss of function mutants) exhibited reduced plastocyanin accumulation even though transcript levels remained high (Abdel-Ghany et al. 2005); supporting that plastocyanin requires Cu for final assembly and for stability, as similarly suggested for *Chlamydomonas* (Li and Merchant 1995). Interestingly, even though both plastocyanin forms are seemingly similar in function (Pesaresi et al. 2008), new evidence suggests that plastocyanin 2 (PC2) accumulates during increased Cu feeding even though photosynthetic benefits were not observed. Plastocyanin could, therefore, have a secondary role as a Cu buffer (Abdel-Ghany 2009). The mechanism for Cu delivery between PAA2 and plastocyanin has not been determined, and a Cu chaperone in the thylakoid lumen has not been identified. It is possible that plastocyanin receives its Cu directly from PAA2 or from a Cu pool. Along with PAA1, another possible Cu transporter in the chloroplast envelope membrane is, HMA1, that may supply some Cu to CSD2 in the stroma (Seigneurin-Berny et al. 2006). Though HMA1, PAA1, and PAA2 are in the HMA family, they do contain some differences. Unlike PAA1 and PAA2, HMA1 does not have conserved MxCxxC *N*-terminal heavy metal binding domains; instead it contains a poly-histidine domain. In addition, HMA1 contains a SPC ion transduction domain instead of CPC found in PAA1 and PAA2 (Axelsen and Palmgren 2001). Chloroplast Cu concentrations and SOD activity levels decrease in *hma1* mutants, and a photo-oxidative stress phenotype was reported when plants were grown in elevated light (Seigneurin-Berny et al. 2006). However, *hma1* plants did not exhibit defects in total plastocyanin levels. Instead, it was suggested that a decrease in CSD2 activity led to the phenotype observed (Seigneurin-Berny et al. 2006). A defect in plastocyanin levels was observed in a *paa1* mutant that also exhibited an electron transport phenotype (Shikanai et al. 2003; Abdel-Ghany et al. 2005). In the case of *paa1*, the phenotype was partially restored by Cu feeding (Shikanai et al. 2003; Abdel-Ghany et al. 2005), but the *hma1* mutant phenotype was not. (Seigneurin-Berny et al. 2006). It is possible that HMA1, with lower Cu transport activity, mediates the delivery of Cu to plastocyanin in *paa1* mutants during Cu feeding, but the link, if any, between HMA1 and Cu transport to plastocyanin is still unclear.

Like plastocyanin, Cu/ZnSODs also require Cu for final assembly, activity, and stability. When Cu delivery to the chloroplast stroma is disrupted by a *paa1* loss of function mutant, CSD2 proteins do not accumulate to wild-type levels while CSD2 transcript levels increase (Abdel-Ghany et al. 2005). The Cu Chaperone for SOD (CCS) delivers Cu to Cu/ZnSODs (Culotta et al. 1997), and is also active in the cytosol and plastids of plants (Chu et al. 2005). The stromal and cytosolic versions of CCS are encoded by one gene in *Arabidopsis* with two in-frame ATG sites that

span a chloroplast transit peptide (Chu et al. 2005). In a T-DNA knock-out mutant of *CCS* (CCS-KO), Cu delivery to Cu/ZnSODs was dramatically reduced and Cu/ZnSOD proteins did not accumulate, further suggesting that Cu/ZnSODs require Cu delivery for protein stability (Chu et al. 2005). It is not known if CCS in stroma acquires Cu directly from PAA1 for delivery to Cu/ZnSOD.

While CSD1 and CSD2 receive their Cu from CCS in the compartment in which they are active, it is likely that CSD3 in the peroxisome does not. CSD3 has a peroxisomal targeting sequence but it likely receives its Cu cofactor in the cytosol prior to import since the peroxisome can import proteins in a folded state. Complementation of a CCS loss of function mutant (CCS-KO) using a CCS version without the chloroplast targeting sequence rescues both CSD1 and CSD3 activities, but not CSD2 in the stroma (Chu et al. 2005).

4.2 Delivery of Cu to other Compartments

4.2.1 Mitochondria

The Cu delivery mechanisms involved in the mitochondria are known mainly for yeast and mammalian cells; however, some homologous proteins have been found in plants. Delivery of Cu to cytochrome *c* oxidase in yeast is accomplished by Cox11, Cox17, Cox19, and Sco1 (Carr and Winge 2003). The mitochondrial matrix in yeast stores Cu as a pool of soluble low molecular weight ligand complexes (Cobine et al. 2004). It was suggested that the Cu pool in the matrix may supply Cu to the intermembrane space and Cox17. Cox17 is a metallo-chaperone that delivers Cu to Cox11 and Sco1 in the intermembrane space, which in turn deliver Cu to different cytochrome *c* oxidase subunits (Horng et al. 2004). *Arabidopsis* functional homologs for AtCOX17 (Balandin and Castresana 2002) and AtCOX19 (Attallah et al. 2007) have been identified. However, homologs of Cox11 and Sco1 chaperones in plants, along with Cu transport across the mitochondria membranes, have not been characterized.

4.2.2 Endomembrane and Secretory Pathway

The RAN1 (HMA7) (responsive-to-antagonist 1) Cu transporter is a functional homolog of yeast and human P-type ATPase Cu transporters active in the endomembrane system (Hirayama et al. 1999). Homologs of RAN1 in yeast and mammals act in Cu transport from the cytosol into the secretory pathway (Lutsenko et al. 2007). A mild loss of function in a *ran1* mutant in *Arabidopsis* lowered the plant's ability to respond to an antagonistic ethylene signal. This evidence suggests that RAN1 is involved in Cu delivery to ethylene receptors (Hirayama et al. 1999). A dramatic loss of RAN1 function led to phenotypes associated with reduced cell

wall elongation (Woeste and Kieber 2000), perhaps due to defects in extracellular Cu oxidases and laccases.

Another member of the HMA family of Cu transporters, homologous to RAN1, is HMA5 (Williams and Mills 2005). HMA5 is mainly localized in root and flower tissues. In *hma5* loss of function mutants, a phenotype associated with cell wall elongation was observed. However, *hma5* had no defects in ethylene reception, as observed for *ran1* (Hirayama et al. 1999; Woeste and Kieber 2000; Andrés-Colas et al. 2006). Together, RAN1 and HMA5 could be involved in supplying Cu to many or all extracellular Cu proteins, but their specificity likely involves organ and intracellular locations relative to where extracellular oxidases and laccases receive their Cu.

5 Senescence, Reallocation, and Delivery to Reproductive Tissues

Copper is not readily reallocated from older leaves to younger tissues. During Cu deficiency, young leaves, shoot meristems, and reproductive tissues are affected before older leaves show signs (Marschner 1995). Therefore, a significant proportion of the Cu allocated to reproductive tissues likely comes directly from the roots (Waters and Grusak 2008). However, there are several indications that some Cu from older leaves is reallocated to newer leaves and reproductive tissues by chelators via symplastic movement. During senescence, the transcripts of the CCH chaperone increase (Mira et al. 2001b). As a chelator with a putative *C*-terminal peptide to facilitate symplastic movement, CCH may chelate Cu in the cytosol during senescence for movement to vascular bundle tissues or movement within the phloem (Mira et al. 2001b; Andrés-Colas et al. 2006). Up-regulation of MT1 has also been reported during senescence (Mira et al. 2002), and it is possible that MT1a and MT2b are involved in phloem reallocation of Cu (Guo et al. 2003).

Another mechanism for reallocating Cu likely involves nicotianamine and the Yellow Stripe-Like (YSL) transporters. In addition to the xylem, nicotianamine is also found in phloem sap. Nicotianamine is a precursor of phytosiderophores which together with YSL transporters is involved in the strategy-II Fe uptake in monocot roots (Briat et al. 2007). Dicots, like *Arabidopsis*, use a strategy-I Fe uptake system that utilizes the root surface FRO2 ferric reductase and the IRT1 ZIP-family transporter, yet *Arabidopsis* encodes eight YSL transporters (Briat et al. 2007). These YSL transporters, likely function to import nicotianamine metal complexes (Schaaf et al. 2004; DiDonato et al. 2004), which could then act as a metal ion redistribution system between tissues via the phloem (Briat et al. 2007; Waters and Grusak 2008). In a *ysl1 ysl3* double loss of function mutant, Cu concentrations in seeds were reduced by 82% when compared to the parental line (Waters et al. 2006). In addition, this mutant line did not efficiently reallocate Cu and Fe from rosette and cauline leaves (Waters and Grusak 2008).

For proper seed set adequate Cu is required (Marschner 1995; Epstein and Bloom 2005). Cu delivery to cells involved in reproduction likely receives most of their Cu directly sent from the roots, but it appears that Cu delivery is via the xylem and the phloem (see above). Cu, along with other metals, bound to nicotianamine could be imported by YSL transporters, and moved through the symplast by CCH. Cells that do not have plasmodesmata for intercellular Cu trafficking by CCH, like pollen, would require COPT1, which is highly expressed in pollen (Sancenon et al. 2004). COPT1 mutant plants with reduced expression exhibit defects in pollen development (Sancenon et al. 2004). Extracellularly, plantancyanin has been implicated in pollen tube guidance (Kim et al. 2003; Dong et al. 2005), and HMA5 is highly expressed in flowering tissues, most likely pollen (Andrés-Colas et al. 2006). HMA5 may deliver Cu to plantacyanin in pollen, but this connection between the two has not been verified experimentally.

6 Regulation of Copper Homeostasis

Plants that are Cu deficient exhibit photosynthetic deficiencies, shoot apical meristem death, curling of leaves, and poor seed set (Marschner 1995; Yruela et al. 1996). To avoid Cu deficiency or toxicity symptoms in a sub-optimal environment, plants are capable of directing Cu delivery based on needs via regulation of Cu delivery systems. Copper toxicity leads to increased expression of some transporters and Cu chelators (see above). However, during Cu deficiency, post-transcriptional regulation of many Cu proteins is mediated by microRNA directed cleavage of Cu protein mRNA. Transcriptional activation of microRNAs, and possibly transporters, during Cu limited growth is mediated by a SPL7 transcription factor. Together, this mechanism to down-regulate Cu proteins and delivery systems may allow for prioritized delivery to the most essential Cu proteins during limited Cu availability.

6.1 Transcription Factors

Transcriptional responses to Cu require transcription factors that can sense Cu. *Chlamydomonas* are capable of switching between two functionally similar photosynthetic proteins, cytochrome *c6* (heme protein) and plastocyanin (Cu-protein), when Cu levels are limited or sufficient, respectively. This switch is mediated by the transcription factor copper response regulator (CRR1) that activates transcription of the cytochrome *c6* when Cu is limited (Kropat et al. 2005). Interestingly, *Crr1* mRNA and expression is not regulated by Cu, suggesting a post-translational change in the CRR1 protein in response to Cu availability (Kropat et al. 2005). While higher plants cannot substitute plastocyanin with cytochrome *c6*, Cu/ZnSOD

and FeSOD regulation exhibit a similar reciprocal expression pattern (Abdel-Ghany et al. 2005; Cohu and Pilon 2007; Yamasaki et al. 2007).

Higher plants contain a homolog to CRR1 know as SPL7 which has recently been shown to be a key regulator of Cu homeostasis by binding to GTAC promoter core motifs (Yamasaki et al. 2009). There are 12 members in the *Arabidopsis* SPL family that contain a conserved SBP (*SQUAMOSA* promoter-binding protein) DNA binding domain and a nuclear localization signal (Cardon et al 1999). SPL transcription factors have been reported to be involved in development and nutrient homeostasis. In *Arabidopsis*, SPL7 likely mediates regulation of some Cu, Zn, and Fe transporters. Wild-type plants increase *COPT1*, *COPT2*, *ZIP2*, *FRO3*, and *YSL2* mRNA when Cu is limited, but in a *spl7* mutant the mRNA of these transporters did not increase (Yamasaki et al. 2009). The *YSL2* promoter contains 5 GTAC core motifs indicating that the transporter may be directly regulated by Cu via SPL7. The Cu-chaperone CCS, which has been shown to decrease during Cu deficiency (Wintz et al. 2003), did not decrease in the *spl7* mutant (Yamasaki et al. 2009). When the *spl7* mutant was grown on low Cu it exhibited a severe growth phenotype, supporting that SPL7 is an important regulator during Cu-limitation. On the other hand, HMA5 and FRO6, which are regulated by Cu, and ATX1 (constitutively expressed), were not identified as being regulated by SPL7 (Yamasaki et al. 2009). It is possible that yet another Cu sensitive regulatory mechanism for HMA5 and FRO6 exists. While SPL7 may regulate some Cu transporters and chaperones directly, SPL7 has also been shown to activate specific microRNA transcription during Cu limited growth, leading to the cleavage of many Cu protein mRNAs (Yamasaki et al. 2009). In the *spl7* mutant, miR397, miR398, miR408, and miR857 (the Cu microRNAs) were not detected even when Cu was limited (Fig. 1).

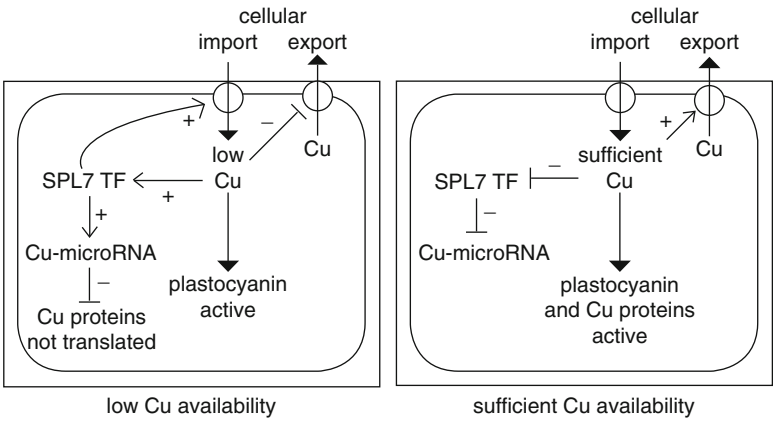

Fig. 1 Model of Cu homeostasis regulation during low and sufficient Cu availability. *SQUAMOSA* promoter-binding protein like-7 transcription factor (SPL7 TF) and open circles for plasma membrane transporters are indicated. Cu uptake, export, and delivery are represented with closed arrow heads. Up-regulation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

6.2 *The Cu microRNAs*

MicroRNAs belong to a highly conserved group of small 20-21-nt RNAs that can disrupt mRNA translation by guiding the cleavage of target mRNAs (Jones-Rhoades and Bartel 2004; Jones-Rhoades et al. 2006). miR398 was the first microRNA shown to target mRNAs that encode Cu/Zn superoxide dismutases in the cytosol (CSD1) and the chloroplast (CSD2) of *Arabidopsis* (Sunkar et al. 2006). Oxidative stress was shown to reduce mature miR398 levels that led to increased CSD1 and CSD2 mRNA and enzyme activity. During non-stress growth conditions, Cu availability was also shown to regulate Cu/ZnSOD expression and activity (Abdel-Ghany et al. 2005; Cohu and Pilon 2007). Linking Cu availability with the regulation of CSD1 and CSD2 by miR398 was established when Cu-supplemented *Arabidopsis* plants, demonstrated an absence of miR398 while CSD1 and CSD2 mRNA abundance increased (Yamasaki et al. 2007). The transcripts of plantacyanin and several members of the laccase family were identified as targets of miR397, miR408, and miR857 directed cleavage during Cu-limited growth (Abdel-Ghany and Pilon 2008). Together these studies suggest that Cu microRNA mediated down-regulation of many Cu-proteins is a mechanism to allow for Cu delivery to the most essential of the Cu-proteins, such as plastocyanin. Another interesting observation of microRNA in Cu homeostasis was the observation that sucrose in tissue culture medium elevated miR398 levels regardless of Cu levels (Dugas and Bartel 2008). This suggests that there is additional regulation on Cu microRNAs from other signaling sources.

SPL7 is mainly found in the roots yet microRNAs are found throughout the plant and sometimes only in above-ground tissues (Yamasaki et al. 2009; Abdel-Ghany and Pilon 2008). High expression of SPL7 in the roots indicates a role in detecting Cu availability at the site of Cu entry, then orchestrating whole plant Cu delivery. Recently, miR398, among other microRNAs, was found in the phloem of *Brassica napus* (Buhtz et al. 2008), rapeseed, and pumpkin (Pant et al. 2008), which suggests that Cu homeostasis signals could originate from source tissues. This method of signal delivery could be very important for young developing leaves during Cu-limitation so that proper Cu delivery to essential Cu-proteins is maintained during initial development.

7 Overview

Transition metal homeostasis is perhaps more completely understood for Cu than any other metal in plants. Identification of Cu transporters and metallo-chaperones that are important in Cu delivery to ethylene receptors, for photosynthesis, and Cu movement into and out of the cell has provided a more complete understanding of Cu homeostasis mechanisms. Information on Cu delivery mechanisms has also allowed for studies that examine how Cu homeostasis is regulated during

development and the changing Cu status, both at a cellular and whole plant level. While Cu delivery mechanisms and regulation of Cu homeostasis is becoming clearer, the biological function of Cu proteins remains unclear in many cases. As the regulation pattern for Cu proteins and delivery mechanisms is resolved, it may help in identifying the biological role of many Cu proteins, both essential and seemingly non-essential.

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