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Copper cofactor delivery in plant cells

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Copper (Cu) is a micronutrient that has roles in photosynthesis, respiration, antioxidant activity, cell wall metabolism and hormone perception. Excess Cu is toxic and therefore its delivery has to be tightly regulated. Recent progress in the study of Cu homeostasis has revealed not only components of the Cu delivery machinery but also regulatory systems that control Cu-protein expression and coordinate the activity of Cu-delivery systems. The response of photosynthetic organisms to Cu deficiency indicates the existence of cross-talk between metal cofactor delivery pathways. Next to its well-established roles in plant metabolism, a novel function for Cu, first discovered in plants, is in the biogenesis of molybdenum cofactor. Defects in Cu delivery factors also suggest important roles for Cu in cell expansion.

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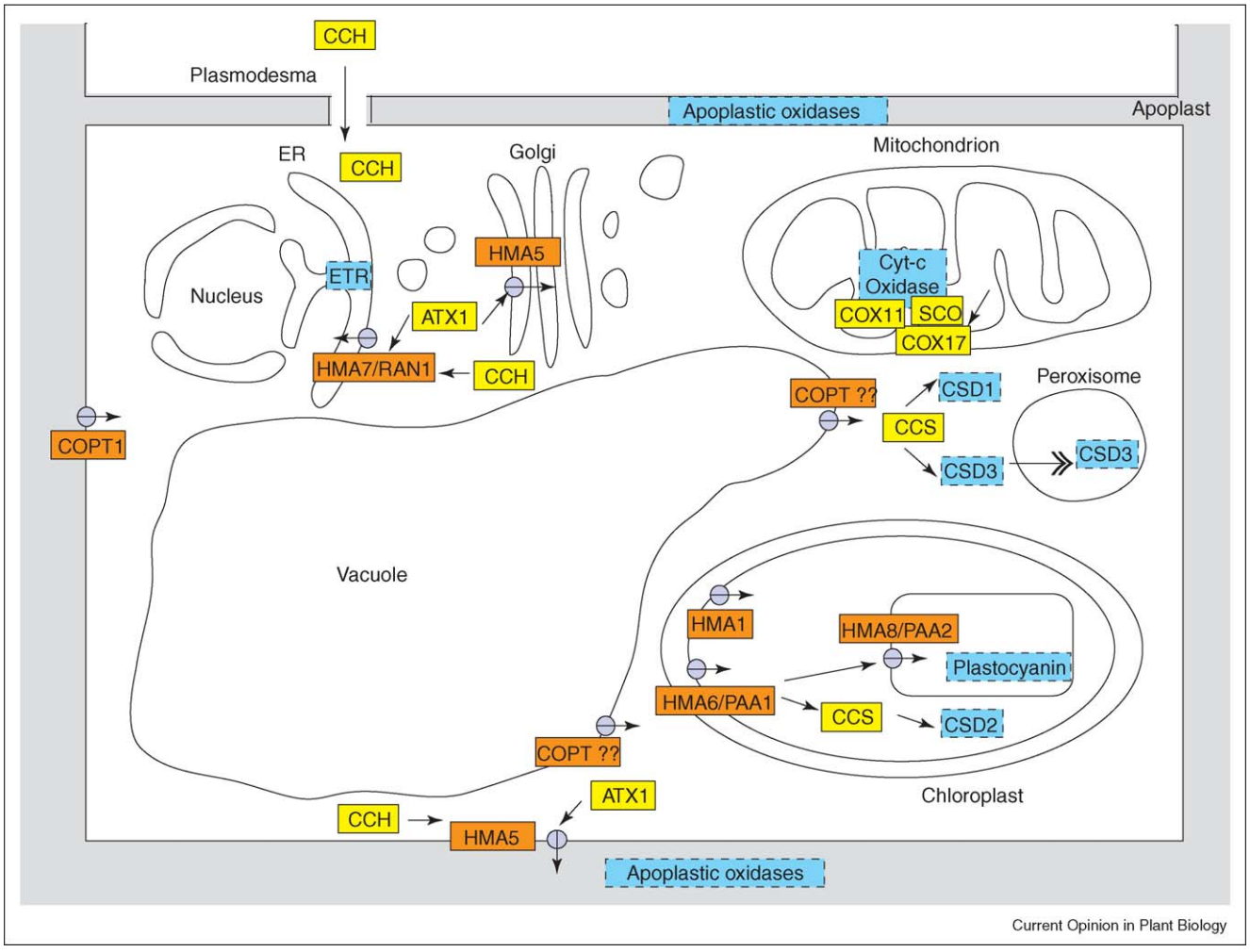
Introduction

In plants, the transition metal copper (Cu) is a cofactor for plastocyanin, copper/zinc superoxide dismutase (Cu/ZnSOD), cytochrome-*c* oxidase, and the ethylene receptors for the apoplastic oxidases: ascorbate oxidase, diamine oxidase and polyphenol oxidase [1]. In the environment of the cell, Cu can exist in two oxidation states, Cu^{2+} and Cu^{+}. Therefore, Cu is redox-active and, while bound to proteins, can be used in electron-transfer reactions. The same redox-activity might, however, also promote the formation of reactive oxygen radicals and therefore the cellular concentration of Cu should be controlled tightly. Copper is classified as a soft metal and prefers to bind with soft ligands. For example, Cu^{2+} is often found to be bound by nitrogen in histidine side chains, whereas Cu^{+} prefers interaction with the sulfur in cysteine or methionine [2]. The Cu-binding affinity of regulatory Cu-binding proteins in both yeast and bacteria is in the femtomolar range, which suggests that the chelating capacity of the cytosol is so high that effectively no free Cu ion is present in the cell [3]. Within the plant cell, Cu is required in at least six locations (see Figure 1): the cytosol, the endoplasmic reticulum (ER), the mitochondrial inner membrane, the chloroplast stroma, the thylakoid lumen and the apoplast [1]. To allow optimal plant growth and development, a balanced delivery of Cu cofactors to each of these destinations is required under varying conditions of supply. This review highlights advances made in the past two years in the fields of Cu delivery and cofactor assembly in plants.

Copper entry into the cytosol

Copper most likely enters the cytosol via a COPPER TRANSPORTER PROTEIN (COPT)-family transporter [4]. The COPT transporters belong to an evolutionary conserved family of transporters called the Copper Transporter family (CTR), which have three transmembrane domains and are characterized by a high methionine content that is thought to play a role in Cu translocation. There are several CTR-like transporters in *Arabidopsis* [4] and, in analogy to the situation in yeast, some family members might be expressed in the plasma membrane whereas others may be active in internal membranes, facilitating release from intracellular stores [5]. It is not yet clear how the COPT or other CTR-like transporters function, but it is likely that Cu enters the cytosol in a reduced form as Cu^{+} [6]. The COPT1 transporter is likely to be active in the cell membrane [7•] and its expression is negatively regulated by copper [4]. The protein is highly expressed in root tips, stomata, trichomes, pollen, and embryos [7•]. All of these cells are characterized by a lack of functional plasmodesmata, which blocks the acquisition of nutrients by a symplastic route. *COPT1* antisense plants have decreased Cu levels as a result of decreased Cu uptake and show sensitivity to Cu chelators. Furthermore, these plants have a pollen-development defect and a root-elongation phenotype, both of which are reversed by Cu feeding [7•]. Both the genes for the COPT family members, *COPT1*, *COPT2*, *COPT3* and *COPT5*, and those for the divalent metal ion transporters *ZIP2* and *ZIP4* complement a yeast *ctr1* deletion mutant that is defective in high-affinity Cu uptake [4,8]. Therefore, two members of the ZIP family might function next to the COPT transporters in Cu uptake in plant cells [8]. Metallothioneins, which are upregulated by Cu stress, do not seem to participate in Cu delivery, but these proteins might buffer cytosolic copper [9].

Figure 1



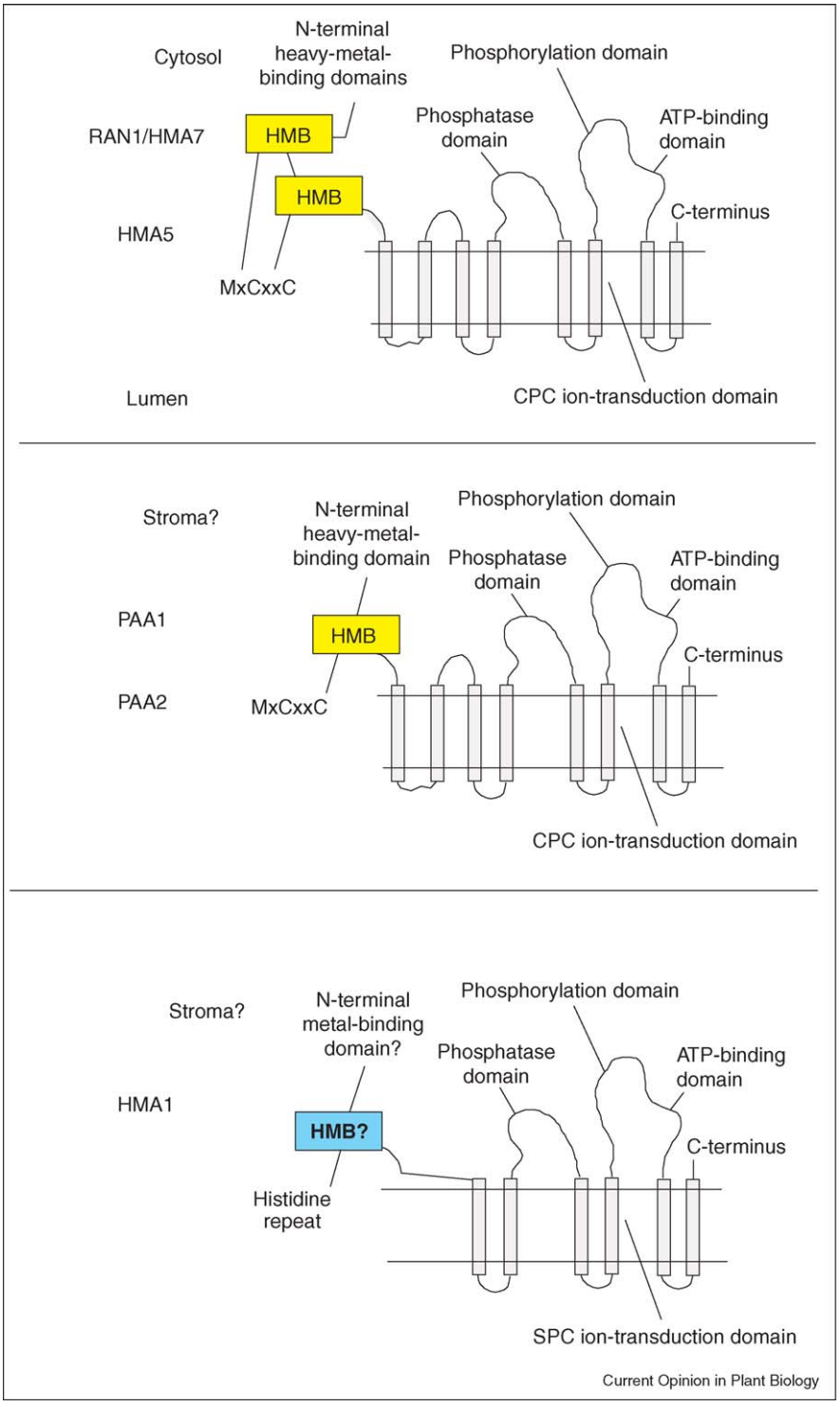
Copper delivery in plant cells. Copper proteins are indicated in blue, transporters in orange and Cu–metallo chaperones in yellow. The most likely location for each transporter protein is indicated but, in most cases, the location shown is not based on direct evidence.

Cu delivery to the endomembrane system and apoplast

The first insight into the targeting of a Cu cofactor to a specific target came from the analysis of mutants in which the *RESPONSIVE TO AGONIST1 (RAN1)/HEAVY METAL ATPase7 (HMA7)* gene is disrupted [10]. The *RAN1* gene encodes a Cu-transporting P-type ATPase that is needed to deliver Cu to the ethylene receptors, which are Cu-binding proteins [11] now believed to be localized in the ER [12]. In *Arabidopsis*, RAN1 was the first functionally characterized heavy metal ATPase (HMA) of the eight-member family [13]. The Cu-transporting branch of the HMA family is characterized by the presence of a CPC (using the single-letter code for amino acids) ion-transduction motif in the sixth predicted transmembrane domain and by one (in PAA1 [for P-type ATPase of *Arabidopsis*1]/HMA6 and PAA2/HMA8) or two (in HMA5 and RAN1/HMA7) heavy-metal-binding domains that have a MxCxxC motif (see Figure 2; [13]). Homologs of RAN1 in yeast and mammalian cells function in the removal of Cu from the cytosol into a secretory compartment or the extracellular space. Mild alleles of *ran1* affect only ethylene signaling [10], but strong loss-of-function alleles also affect ethylene-independent processes including cell elongation [14]. *ran1* mutants do not seem to be Cu sensitive, instead their phenotypes are suppressed by Cu feeding.

There is a close homolog of *RAN1*, called *HMA5*, in *Arabidopsis* [13]. The *HMA5* gene is expressed mainly in roots and flowers [15••]. The *hma5* T-DNA insertion mutants are Cu sensitive and have wave-like root growth and growth arrest. This phenotype is the opposite of that observed for the COPT antisense lines, supporting the notion that COPT1 and HMA5 transport Cu in opposite directions. *hma5* mutants do not display the defects related to ethylene signaling that are noted for *ran1*

Figure 2



Schematic structure of Cu-transporting P-type ATPases. A domain structure for the five heavy-metal-transporting ATPases that have an implied role in Cu delivery is given. The indicated topology is based on predictions but has not been verified experimentally.

[10,14,15••]. However, both *ran1* and *hma5* loss-of-function mutants have phenotypes that are associated with cell expansion [14,15••]. It is possible that *ran1* and *hma5* mutations affect apoplastic oxidases, which require copper and could function in cell expansion. The activities of ascorbate oxidase, polyphenol oxidase, and amine oxidase have not, however, been reported for the *ran1* and *hma5* mutants. Recent reports indicate roles for apoplastic ascorbate oxidase in cell expansion, plant biomass production and salt tolerance [16,17], suggesting that the link between Cu and cell expansion merits further exploration. With respect to the phenotype of *hma5*, it is interesting to note that a protein of the multicopper oxidase family plays a role in directional root growth [18]. Another Cu protein in the apoplast is plantacyanin, which plays a role in pollen-tube guidance on the stigma [19,20] and could be affected by the COPT, HMA5 or RAN1 transporters. The specific roles of RAN1 and HMA5 are probably related to their intracellular locations, which have not been determined. On the basis of the mutant phenotypes, however, it seems likely that RAN1 is active in an endomembrane system compartment, perhaps the ER, whereas HMA5 might be in the plasma membrane or a late secretory compartment (see Figure 1).

Cytosolic metallochaperones and functions for Cu in the cytosol

The RAN1 and HMA5 transporters are related in sequence to a yeast P-type ATPase called CCC2. This protein is required to deliver Cu to an endomembrane compartment for the activation of a multicopper ferroxidase, which is required for iron acquisition at the cell surface. In yeast, Ccc2p interacts with a small cytosolic Cu chaperone called Antioxidant1p (Atx1p), which delivers Cu to the transporter by direct protein–protein contact [21]. The structure of ATX1p is very similar to that of the amino-terminal heavy-metal-binding domain of the Cu-transporting ATPases [22]. *Arabidopsis* has two homologs of yeast *ATX*, called COPPER CHAPERONE (CCH) [23] and ATX1 [15••]. The carboxy-terminal of the *Arabidopsis* CCH protein is extended relative to that of yeast and *Arabidopsis* ATX1 proteins [24]. Both the CCH and ATX1 proteins complement the yeast *atx1* mutant and interact with the amino-terminus of HMA5, as shown by yeast two hybrid assays [15••,23]. It was shown in the yeast-two hybrid assay, however, that the carboxyl terminus of CCH has a negative effect on its interaction with HMA5 [15••]. The expression of *CCH* is upregulated by Cu stress and during senescence, a time when the plant reallocates nutrient resources [25]. The gene is expressed in areas around the vascular tissue and the protein was detected in phloem [26•]. The carboxy-terminal domain of CCH could be involved in the transport of the protein through plasmodesmata to non-nucleated cells, such as sieve elements, thus providing a symplastic pathway for intercellular Cu transport [26•]. Homologs of *ATX1* and the endomembrane P-type ATPases have been found in the green algae *Chlamydomonas* [27] where, as in yeast, they have a function in iron acquisition by providing Cu to a multicopper ferroxidase that is required for Fe uptake [28]. Fe uptake in higher plants uses a mechanism which differs from that in algae and yeast, and there is no indication that *RAN1* or *HMA5* function in Fe uptake in *Arabidopsis*. However, the phenotypes of *cch* and *atx1* loss-of-function mutants have not yet been described.

Copper is also a cofactor, together with Zn, for cytosolic Cu/ZnSOD. Because the biogenesis of this protein in plants is linked to plastid Cu metabolism it will be discussed below. An exciting recently discovered role for Cu is in the synthesis of a molybdenum cofactor (see Figure 3; [29••]). This observation now links Cu metabolism to nitrogen assimilation and phytohormone biosynthesis [30].

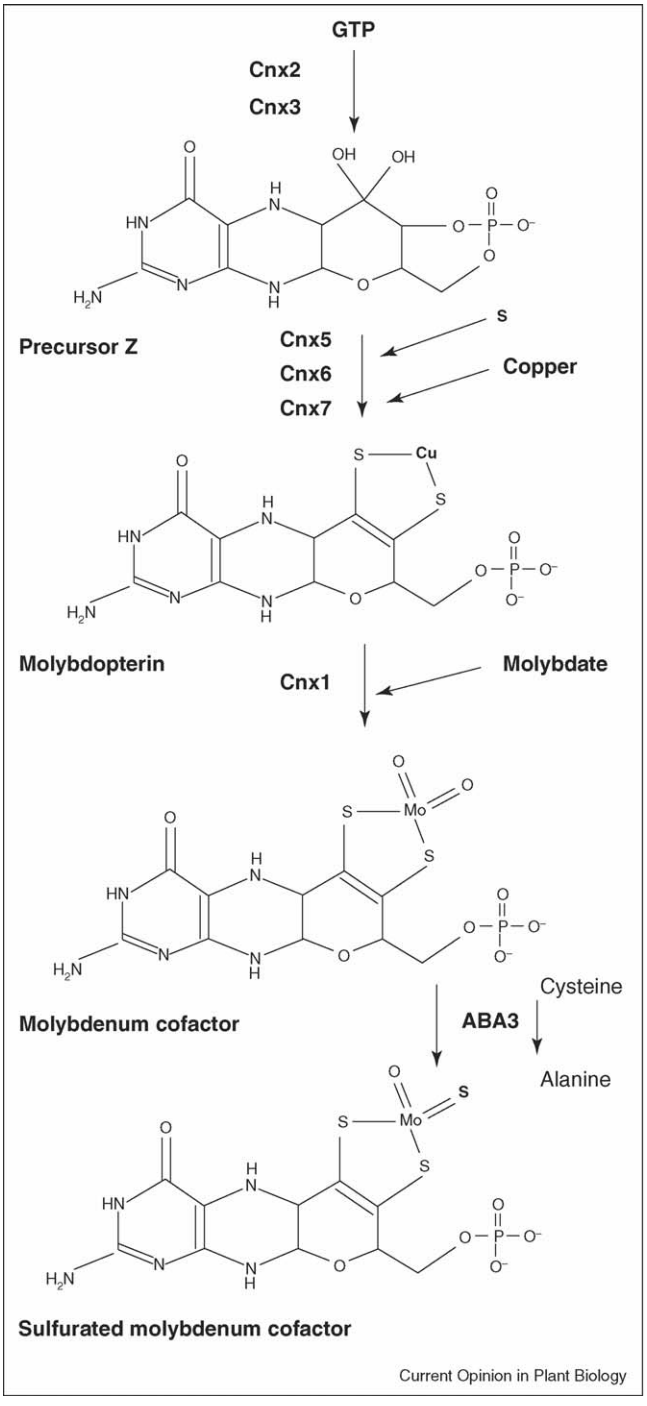
Copper delivery to mitochondria

An important Cu-containing protein in mitochondria is cytochrome-*c* oxidase, the terminal oxidase in the inner membrane. This multi-subunit protein contains three Cu ions as cofactors in two Cu sites in addition to heme [31]. Genetic screens in yeast have identified assembly factors that are required for the formation of a functional cytochrome-c oxidase. Three of these proteins, Sco1p, Cox11p and Cox17p, are metallo chaperones that bind Cu through thiols [31]. The Cox17p chaperone delivers Cu to Sco1p and Cox11p [32], which in turn insert Cu into the A and B Cu sites [31]. Some older models suggested that Cox17p could pick up cytosolic Cu, somehow diffuse into the intermembrane space and deliver Cu to Sco1 and Cox11p. Interestingly enough, however, Cox17p is active when tethered in the mitochondrial intermembrane space [33•]. The matrix, where Cu seems to be stored in a low-molecular-weight form, is the source of Cu for Cox17p [34••]. How Cu enters the matrix and how it is exported is not yet clear. *SCO1*, *COX11* and *COX17* are all conserved and homologs are found in plants; plant COX17 complements a *cox17* yeast mutant [35]. Yeast is probably the best model system for studies of the basic mechanisms of cytochrome-*c* oxidase assembly, but some interesting questions might need to be addressed in plants. For instance, how do higher plants prioritize Cu delivery to plastids, mitochondria and other intracellular targets under Cu deficiency?

Copper delivery to plastocyanin and superoxide dismutase

PAA1 and *PAA2* encode copper-transporting P-type ATPases that are located in the chloroplast envelope inner membrane and thylakoids, respectively [36,37••]. Characterization of *paa1* and *paa2* mutants showed that the two transporters have distinct functions. Both transporters are required for copper delivery to plastocyanin and for efficient electron transport, but copper delivery to the stroma is only inhibited in *paa1* and not in *paa2* mutants. Thus PAA1 and PAA2 function sequentially

Figure 3



A novel role for Cu in molybdenum cofactor synthesis. Plants have only four Mo-requiring proteins: nitrate reductase, xanthine dehydrogenase, and aldehyde oxidase are active in the cytosol, whereas sulfite oxidase is most probably active in peroxisomes [41]. With the exception of bacterial nitrogenase, all biologically active Mo occurs in a special pterin-derived cofactor, termed molybdenum cofactor or Moco, which is assembled in the cytosol [42]. Studies performed in plants have significantly contributed to what we know about Moco synthesis, and this is now one of the best-understood cofactor assembly systems [42]. Seven enzymes are required for Moco synthesis in plants. The insertion of Mo in the molybdopterin skeleton requires the CNX1 protein. The structure of the molybdopterin-bound form of CNX1 revealed two surprises [43]. First, the enzyme produced an adenylated intermediate, molydopterin-AMP, which was found bound to the enzyme. Second, Cu was bound to the thiols of the molybdopterin moiety, and it was suggested that the presence of Cu served to protect the thiols and facilitate Mo insertion. A new role for Cu has thus been found in the synthesis of Moco.

in copper transport through the envelope and thylakoid membrane, and are functional homologs of the cyanobacterial Cu-transporting ATPases CtaA and PacS [38]. A *paa1 paa2* double mutation was seedling lethal [37••], underscoring the importance of Cu to photosynthesis and the essential role of plastocyanin for photoautotrophic growth in *Arabidopsis* [39]. Interestingly, the phenotypes of *paa1* mutants and to a lesser extent *paa2* mutants, but not of the *paa1 paa2* double mutant, were alleviated by Cu feeding, suggesting that an alternative lower-affinity pathway for Cu delivery exists [36,37••]. A candidate for the alternative transporter in the envelope is HMA1, which was localized in envelopes and affects Zn- and Cu-uptake activity when expressed in yeast [40••]. A *hma1* T-DNA insertion mutant showed no defect in plastocyanin but was defective in total plastid SOD activity. Like *paa1* mutants, the *hma1* plants have reduced Cu levels in plastids but have total leaf levels of Cu that are comparable to those of wildtype plants. The HMA1 transporter does not have a canonical amino-terminal heavy-metal-binding domain and the sequence in its fourth transmembrane domain, which corresponds to the sixth transmembrane domain of PAA1 and PAA2, is SPC instead of CPC (see Figure 2). It is possible that HMA1 transports divalent ions, including Cu^{2+}, and the Cu and/or Zn that is imported by HMA1 might be preferentially targeted to Cu/ZnSOD in the plastids. The notion that Cu^{2+} could be present in the space between the two envelopes is supported by the presence of the Cu^{2+}-binding protein AtCutA in this location [41]. In thylakoids, the alternative transport activity might be explained by the energy-independent Cu^{2+} transport that was detected using a fluorescence-quenching assay [42], but the component responsible for this activity is not yet described.

Arabidopsis has three isoforms of Cu/ZnSOD: cytosolic (CSD1), chloroplastic (CSD2), and peroxisomal (CSD3). In yeast and mammals, the maturation of Cu/ZnSOD requires a Cu-chaperone called Copper Chaperone for SOD (CCS). This protein has an amino-terminal ATX-like heavy-metal-binding domain, a central region with similarity to its target Cu/ZnSOD, and a carboxy-terminal domain that has additional cysteine residues. The CCS protein is thought to transfer Cu to its target by direct protein interaction and to play a role in SOD activation by catalyzing the formation of a disulfide bond [43,44]. *Arabidopsis* has just one functional homolog of the yeast CCS [45], yet there are three subcellular locations for Cu/ZnSOD isoforms, the cytosol, the plastid and the peroxisome. When the full-length *Arabidopsis* protein is fused to green fluorescent protein (GFP) it localizes to chloroplasts

only [45], but a T-DNA insertion in CCS affects all three Cu/ZnSOD activities [46••]. It is likely that CCS is active both in plastids, delivering Cu to CSD2, and in the cytosol, delivering Cu to both CSD1 and CSD3. CSD3 has a peroxisomal-targeting sequence and might subsequently be imported by the peroxisomes, which can import proteins in a folded state. The cytosolic CCS activity in plants might result from the use of an alternative translational start site that skips the chloroplast-targeting peptide [45,46••]. Expression of the CCS protein without its transit sequence in the T-DNA mutant only rescues the cytosolic and peroxisomal Cu/ZnSOD activities. It should be noted that the *ccs* T-DNA lines were not fully devoid of Cu/ZnSOD activity, and that these plants had very mild growth phenotypes compared to a knockdown mutant that had reduced stromal Cu/ZnSOD activity [47]. Although yeast Cu/ZnSOD has an absolute requirement for CCS, *Caenorhabditis elegans* and mammalian Cu/ZnSOD can also be activated by a CCS-independent pathway that uses glutathione [48]. A similar mechanism might operate in the *Arabidopsis ccs* mutant [46••].

Plants have an iron superoxide dismutase (FeSOD) in the stroma of the chloroplast. Interestingly, Cu availability in the chloroplast regulates the activity of the stromal SOD isoforms [37••]. At low Cu concentrations, the FeSOD is active and Cu/ZnSOD and CCS expression is shut off, so Cu is preferentially targeted to plastocyanin in the thylakoid lumen. At higher Cu concentrations, FeSOD expression is shut off, possibly saving Fe for other uses, and Cu/ZnSOD becomes active in the stroma and cytosol. The effects of Cu feeding and of *paa1* and *paa2* mutations on the transcript levels of superoxide dismutase genes and *CCS* strongly suggest that stromal copper levels regulate the expression of these nuclear genes [37••,45]. Thus, a signaling pathway must exist that senses Cu levels in the chloroplast and affects the expression of nuclear genes.

Plastocyanin is indispensable in plants [39] and therefore a priority for Cu delivery. In many algae including *Chlamydomonas*, however, a Cytochrome-c6 can functionally replace plastocyanin under low Cu conditions, presumably saving Cu for other essential functions, such as respiration, that take priority under these conditions [49]. A Cu/ZnSOD is not found in cyanobacteria or in the eukaryotic green alga *Chlamydomonas reinhardtii*, which depend on FeSOD and MnSOD activities. Thus, Cu delivery pathways in higher plants and algae might have adapted differently to ensure the delivery of Cu to the most essential Cu proteins, which are different in each organism.

Perspective and conclusions

Early *in vitro* studies on cofactor assembly with purified proteins indicated that metal insertion is energetically favorable. Cofactor insertion seemed to be spontaneous, with selectivity provided by the affinities and geometry of binding ligands. Recent progress has clarified that, in the case of copper and other metals except Zn, cofactor delivery is not always spontaneous and is often regulated by protein interactions. Why would there be a need for metallochaperones in some cases but not in others? One explanation is that metallochaperones help to overcome the high metal-ion-chelating capacity of the cell. This could be the function for ATX1 as it delivers Cu to RAN1 and HMA5 by protein–protein interaction and using a shallow gradient in the binding constants for Cu [3]. Alternatively, metallochaperones provide the cell with a mechanism to control specific delivery pathways in response to metal ion supply. This is demonstrated by the expression pattern of *CCS* in response to Cu deficiency in *Arabidopsis*. Finally, metallochaperones can, as their name implies, prevent inappropriate interactions. This function is best illustrated by the situation in cyanobacteria in which three P-Type ATPases are present in the cell membrane, functioning in the uptake of Cu (CtaA) or in the the extrusion of Zn (ZiaA) or cobalt (CoaT) [50••]. A fourth transporter, PacS, delivers Cu to plastocyanin in the thylakoids [50••]. An Atx-like chaperone [51] functions to deliver Cu to PacS but there is no metallochaperone for delivery to ZiaA. The amino-terminal domains of PacS and ZiaA bind their cognate metal ion, but Cu binds more strongly to the ZiaA amino-terminal domain than does Zn [50••]. The ZiaA amino terminus cannot accept Cu from cyanobacterial Atx because the proteins lack a complementary surface, and therefore Atx both prevents an undesired interaction and facilitates delivery to the correct transporter [50••].

Both the mitochondria and plastids are Cu sinks, but there seem to be no systems for the delivery of Cu to the surface of these organelles. How then is Cu delivery to these organelles feasible? It should be noted that schematic figures of cells, such as Figure 1, are by necessity static and do not usually do justice to the true volume of the vacuole. In reality, the organelles of a plant cell will almost always be very close to the tonoplast. Therefore, the vacuole could be a delivery pathway within the cell, and not just a sequestration compartment. A metal ion might need to diffuse just a short distance away from the tonoplast before encountering another organelle. This notion is supported by most thin-section electron micrographs of plant cells. The analysis of double mutants that are disrupted in genes that encode delivery proteins, together with measurements of the size of the intracellular Cu pool, presents a promising avenue along which to gain further insight into the regulation of Cu delivery in the cell.

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