

## REVIEW:

### COPPER IN PLANTS: ACQUISITION, TRANSPORT AND INTERACTIONS

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#### ABSTRACT

Copper is an essential metal for plants. It plays key roles in photosynthetic and respiratory electron transport chains, in ethylene sensing, cell wall metabolism, oxidative stress protection and biogenesis of molybdenum cofactor. Thus, deficiency in the copper supply can alter essential functions in plant metabolism. On the other hand, copper during decades has been used in agriculture as an antifungal agent and it is also extensively released into the environment by human activities that often cause environmental pollution. Accordingly, excess copper is present in certain regions and environments, and exposure to that can be potentially toxic to plants causing phytotoxicity by the formation of reactive oxygen radicals that damage cells or by the interaction with proteins impairing key cellular processes, inactivating enzymes and disturbing protein structure. Plants have a complex network of metal trafficking pathways in order to appropriately regulate copper homeostasis in response to environmental copper level variations. Such strategies must prevent accumulation of the metal in the freely reactive form (metal detoxification pathways) and to ensure proper delivery of this element to target metalloproteins. The mechanisms involved in the acquisition and the distribution of copper have not been clearly defined although emerging data in last decade, mainly obtained on copper uptake, and both intra- and intercellular distribution, as well as on long-distance transport, are contributing to the understanding of copper homeostasis in plants and the response to copper stress. This review gives a brief overview of the current understanding of main features concerning copper function, acquisition and trafficking network as well as interactions between copper and other

elements.

Keywords: deficiency, regulation, response, trafficking network, tolerance, toxicity

## 1 INTRODUCTION

Plants require mineral nutrient elements, predominantly acquired from the soil but also from foliar applications, to maintain normal growth and development and ensure the completion of life cycles. The acquisition and distribution of these elements are important targets for research because their metabolic and biochemical functions are associated with all aspects of plant physiology, plant biochemistry and plant molecular biology. Copper (Cu) is a redox-active transition metal essential for plants as well as for all living organisms. Cu participates in many physiological processes because it is able to exist in multiple oxidation states *in vivo*. Under physiological conditions Cu exist as  $\text{Cu}^{2+}$  and  $\text{Cu}^{+}$ . The cation  $\text{Cu}^{2+}$  is often bound by nitrogen in histidine side chains, whereas  $\text{Cu}^{+}$  prefers interaction with the sulphur in cysteine or methionine. Cu acts as structural element in certain metalloproteins, many of which are involved in electron transport in chloroplasts and mitochondria as well as in oxidative stress response. Cu ions act as cofactor in enzymes such as Cu/Zn-superoxide dismutase (Cu/ZnSOD), cytochrome c oxidase, ascorbate oxidase, amino oxidase, laccase, plastocyanin and polyphenol oxidase. At cellular level, Cu plays an essential role in cell wall metabolism, signalling to the transcription and protein trafficking machinery, oxidative phosphorylation, iron mobilization and the biogenesis of molybdenum cofactor (for reviews see Raven et al. 1999; Yruela 2005; Gratão et al. 2005; Pilon et al. 2006; Krämer and Clemens 2006; Puig et al. 2007).

Thus, plants require Cu for normal growth and development, and when this ion is not available, plants develop specific deficiency symptoms, most of which affect young leaves and reproductive organs. On the other hand, the redox properties that make Cu essential element also

contribute to its inherent toxicity. Redox cycling between  $\text{Cu}^{2+}$  and  $\text{Cu}^{+}$  can catalyze the production of highly toxic hydroxyl radicals, with subsequent damage to cells at level of lipids, membranes, nucleic acids, proteins and other biomolecules (Halliwell and Gutteridge 1984). Although Cu usually binds to proteins it has capacity to initiate oxidative damage and interfere with important cellular processes such as photosynthesis, pigment synthesis, plasma membrane permeability and other metabolic mechanisms, causing a strong inhibition of plant development (van Assche and Clijsters 1990; Marschner 1995; Küpper et al. 2003; Bertrand and Poirier 2005; Yruela 2005). Cu in excess can become extremely toxic causing symptoms such as chlorosis and necrosis, stunting, and inhibition of root and shoot growth. At cellular level, excess Cu can inactivate and disturb protein structure as a result of unavoidable binding to proteins. Toxicity may result from: *i*) binding to sulfhydryl groups in proteins, thereby inhibiting enzyme activity or protein function; *ii*) induction of a deficiency of other essential ions; *iii*) impaired cell transport processes; *iv*) oxidative damage.

Nevertheless, either Cu deficiency or excess Cu can cause disorders in plant growth and development by adversely affecting important physiological processes in plants. For healthy plant growth and development Cu must be acquired from the soil, transported throughout the plant, distributed and compartmentalized within different tissues and its content carefully regulated within different cells and organelles. Tissue and cellular concentrations of Cu need to be controlled within a narrow physiological range. For this purpose, plants –like all other organisms- have homeostatic mechanisms to acquire appropriate amounts of Cu in diverse environmental conditions and precisely delivering it to specific compartments and target to metalloproteins while avoiding its toxic effect. Thus, the acquisition and assimilation of Cu must be coordinated with mineral supply and plant demand in a complex and regulated interacting network. Cu homeostasis processes are dynamic in nature and respond to metal availability, annual cycles, and growth phases.

Although the mineral nutrition of higher plants is of fundamental importance to agriculture and human health, many basic questions remain unanswered, particularly in relation to the

accumulation of essential heavy metals. Which mechanisms explain that all tissues receive an adequate supply of the heavy metals required for vital cellular processes? Which mechanisms prevent plants from accumulating to toxic levels? These are some questions of fundamental importance in plant biology, which underlie an emerging area of research now that the complete sequencing of several genomes and the necessary molecular tools are available. Genomic approaches are being applied to understand the processes of nutrient acquisition, assimilation and metabolism. In particular, the studies developed in the yeast *Saccharomyces cerevisiae* have contributed to progress in the knowledge of basic cellular components of Cu homeostasis in eukaryotic organisms. The use of genetic and molecular techniques such as sequence comparison to identify transporters, functional complementation of yeast mutants and plant transformation to regulate gene activities has been crucial for this development. A wide range of gene families and proteins are being identified in plants that are likely to be involved in Cu homeostasis. Cu homeostasis is also receiving a growing interest in plant research since it is implicated in adaptive responses to the oxidative damage produced by environmental stress. Mechanisms must exist to satisfy the requirements of cellular metabolism and at the same time to protect cells from toxic effects. At cellular level, specific transporters are responsible for the uptake and secretion of metal ions, and there may be additional transporters that allow sequestration into organelles. In particular, the interaction of metal chaperones with transporters deserves attention since this may have important implications for sequestration of metals within intracellular stores. During the last ten years a rapid progress has been made in this area. Thus, heavy metal homeostasis is a very exciting and fast developing field in plant biology.

This review gives a briefly overview of the current understanding of main features concerning to Cu acquisition, trafficking network and interactions between Cu and other metal ions as well as Cu regulatory and tolerance mechanisms.

## 2 COPPER FUNCTIONS, ACQUISITION AND TRANSPORT

### 2.1 Copper bioavailability.

Cu concentration in vegetative plant tissues varies depending on plant species or ecotypes, developmental stage and environmental factors such as nitrogen supply and soil chemical properties. For instance: *i*) plants grown under high nitrogen supply require significantly more Cu; *ii*) Cu bioavailability tends to be larger in acidic soils. It has been reported that the Cu concentration in plant tissues is between 1 and 5  $\mu\text{g g}^{-1}$  dry weight (Marschner 1995) and the average composition of Cu in leaves is 10  $\mu\text{g g}^{-1}$  dry weight (5-20  $\mu\text{g g}^{-1}$  dry weight) (Baker and Senef 1995) but these concentrations can vary among plant species and varieties. Cu concentrations in cells need to be maintained at low levels since this element is extremely toxic in view of its high redox properties. The critical free Cu concentration in the nutrient media (below which Cu deficiency occurs) ranges from  $10^{-14}$  to  $10^{-16}$  M. Plants usually find a variable supply of Cu in the soil since typically soil solution concentrations range from  $10^{-6}$  to  $10^{-9}$  M (Marschner 1995), but plants may still need to solubilize and reduce the metal.

Concentrations of free metal ions or metal chelates in the soil solution are generally rather low although this depends on soil properties (Kochian 1991; Marschner 1995). In both soil solution and solid phase Cu is mainly associated with inorganic and organic matter by complexation or adsorption. Cu ions have a high affinity for binding sites of soil components, as well as can be adsorbed onto surfaces of clays and Fe or Mn oxides, co-precipitated with carbonates and phosphates or present in the lattice of primary silicate minerals. Cu ions can be also bound to cell walls and to the outer membrane surface of plant root cells. The distribution of Cu among these various solid and plant components will greatly influence the chemical mobility and hence the amount of Cu potentially taken up by plants. At acidic pH, dissolved Cu will increase because of its weaker adsorption and so will increase the free Cu ion activity. Additionally, with increasing pH,

competitive adsorption will arise between organic matters in the solid phase and dissolved organic carbon, generally leading to an increase in Cu concentration in the soil solution due to an increase of dissolved organic carbon (Carrillo-González et al. 2006). Thus, upon increasing pH, the Cu ions activity considerably will decrease at the expense of organically bound complexes species in the soil solution (Sauvé et al. 1997).

On the other hand, in the rizhosphere, root and microbial activities can influence the chemical mobility of metal ions and ultimately their uptake by plants as consequence of alterations of soil pH or dissolved organic carbon (Hinsinger and Courchesne 2007). For instance, in the case of *Graminaceous* species, the increased root secretion of Fe-chelating compounds (phytosiderophores) under Fe deficiency has been reported to increase Cu uptake in a calcareous soil (Chaignon et al. 2002). It is noticeable that soil chemical properties can differ between the bulk soil and the rizhosphere, so considering only properties in the bulk soil might be a poor predictor of Cu bioavailability and ultimately Cu uptake which rather depends on the particular properties induced by roots in the rizhosphere. Accordingly, contradictory results concerning the effect of pH on Cu uptake by plants are found in the literature. In very acidic soils, plant Cu concentration increased compared to calcareous soils in rape (*Brassica napus* L.) and tomato (*Lycopersicon esculentum* L.) (Chaignon et al. 2003; Cornu et al. 2007). On the contrary, Cu accumulation in maize (*Zea mays* L.) was as high in calcareous soils as in acidic soils (Brun et al. 2001). Michaud et al. (2007) did not found a clear relationship between Cu uptake and soil pH in durum wheat (*Triticum turgidum durum* L.) in Cu-contaminated soils, probably due to the implication of root-induced changes of pH and dissolved organic carbon in the rizhosphere. At low pH, alkalization in the rizhosphere was observed compared with the bulk soil, which may result in a reduced Cu bioavailability. In calcareous soils, a larger chemical mobility may be related to phytosiderophore secretion leading to greater Cu uptake in plants.

## 2.2 Copper function in plants

Within the plant cell, Cu is required in at least six locations: the cytosol, the endoplasmic reticulum (ER), the mitochondrial inner membrane, the chloroplast stroma, the thylakoid lumen and the apoplast (Marschner 1995). The number of Cu-dependent proteins in plants is generally smaller compared with other metal-dependent proteins (metalloproteins). In *Arabidopsis* proteome can be found 105 and 21 proteins searching “copper protein” and “copper-binding protein” terms, respectively (Krämer and Clemens 2006). The most abundant Cu proteins in green tissues are plastocyanin and Cu/ZnSOD. In *Arabidopsis* Cu/ZnSOD is present in three isoforms, of which the major isoforms are found in the cytosol (CSD1) and chloroplast stroma (CSD2), and the third isoform is found in peroxisome (CSD3) (Kanematsu and Asada 1989; Bueno et al. 1995). In maize four cytosol Cu/ZnSOD isoenzymes have been found (Kernodle and Scandalios 2001).

In addition to plastocyanin and Cu/ZnSOD there is a large number (>32) of related proteins (blue-copper binding proteins) with unknown functions encoded in the *Arabidopsis* genome (Nerissian et al. 1998). For instance, the existence of a Cu protein involved in photosynthetic reactions of photosystem II (PSII) non-dependent of plastocyanin was reported earlier (Lightbody and Krognann 1967; Barr and Crane 1976). More recently, Burda et al. (2002) found that Cu in an equimolar concentration to PSII reaction centre stimulated *in vitro* the oxygen-evolution activity of PSII. Nevertheless, little information respect to this event exists *in vivo*. An important characteristic of Cu<sup>+</sup> is its ability to bind small molecules such as O<sub>2</sub> as ligands. Thus, Cu is a cofactor of a large number of oxidases. The best-known oxidase is the mitochondrial cytochrome c oxidase. Other members of this enzyme group are: *i*) amine oxidase enzymes associated to the cell wall that catalyzes the oxidation of putrescine that produces H<sub>2</sub>O<sub>2</sub> involved in lignification, cross-linking of cell wall proteins and programmed cell death (Moller and McPherson 1998); *ii*) multi-copper oxidases such as ascorbate oxidases that localize in the apoplast and regulate its redox state, and

laccases also localized in the apoplast but not functionally well understood although a role in lignification has been proposed (Ramocho et al. 2002); *iii*) multi-copper oxidase-like proteins such as SKU5, which are involved in cell wall formation (Sedbrook et al. 2002); *iv*) polyphenol oxidase found in the thylakoids of some plants, such as spinach (Kieselbach et al. 1998) but not in other species such as *Arabidopsis* (Schubert et al. 2002) that is involved in ROS defence.  $\text{Cu}^+$  can also bind ethylene. Accordingly, the ethylene receptor ETR1, which localizes in the endoplasmic reticulum (ER), is dependent on  $\text{Cu}^+$  (Rodriguez et al. 1999). Recently, the role of Cu in the synthesis of a molybdenum cofactor has been proposed (Kuper et al. 2004). This observation now links Cu metabolism to nitrogen assimilation and phytochrome biosynthesis (Mendel 2005).

### 2.3 Copper acquisition and transport

Cu acquisition and transport into and within cells is relatively little known in plants but in the last ten years rapid progress has been made to understand these processes within plant cells, particularly with the application of the knowledge in yeast to other eukaryotes organisms. Consequently, several families of heavy metal transporters involved in intracellular homeostasis have been identified in plants (for reviews see Fox and Guerinot 1998; Himmelblau and Amasino 2000; Williams et al. 2000; Markossian and Kurganov 2003; Krämer and Clemens 2006; Colangelo and Guerinot 2006; Puig et al. 2007). However little results have been obtained respect to long-distance transport or transport processes taking place at root level. For instance, at present it is not clear how plant roots actively mobilize Cu ions. Phytosiderophore secretion by monocots is known to enhance Cu mobilization (Römheld 1991) but there is no evidence for the uptake of Cu-phytosiderophore complexes by plant roots. The recent progress made on the Cu acquisition, transport and distribution is presented here (Fig. 1, Table 1).



### 2.3.1 COPT copper transporters

COPper Transporter Protein (COPT) family has been identified in plants by sequence homology with the eukaryotic Cu transporters named Ctr or by functional complementation in yeast (for reviews Peña et al. 1999; Labbé and Thiele, 1999; Harris 2000; Puig and Thiele 2002; Puig et al. 2007). The *Arabidopsis* genome contains six genes encoding COPT transporters, COPT1-6. The first one, COPT1, is the best characterized member of this Cu transporter family. It was identified by the ability of its cDNA to functionally complement a *Saccharomyces cerevisiae ctr1Δ* mutant defective in high-affinity Cu uptake. COPT1 transporter allows the entrance of Cu into cells from the exterior to the cytoplasm (Kampfenkel et al. 1995; Sancenón et al. 2003). All members of this protein family contain three predicted transmembrane (TM) segments and most possess an N-terminus methionine- and histidine-rich putative metal binding domains (Puig and Thiele 2002; Klomp et al. 2003) (Fig. 2). Genetic data and *in vivo* uptake experiments have demonstrated that an extracellular methionine residue, located approximately 20 amino acids before TM1, and an MxxxM motif within TM2, are essential for Cu acquisition, and probably mediate metal coordination during transport. A symmetrical trimer organization with a novel channel-like architecture has been shown in the human Ctr1 transporter homolog to COPT members (Aller et al. 2004; Aller and Unger 2006).

Metal competition experiments suggest that *Arabidopsis* COPT1, as for other Ctr1 family members, is a high-affinity transporter with specificity for Cu<sup>+</sup> ion (Sancenón et al. 2003) with a  $K_m$  in the lower micromolar range (Eisses and Kaplan 2002; Lee et al. 2002). COPT transporters do not use ATP for Cu import, but their transport ability is stimulated by extracellular K<sup>+</sup>. The COPT1 transporter is likely to be active in the cell membrane and its expression is negatively regulated by Cu. The *COPT1* gene is highly expressed in embryos, trichomes, stomata, pollen and roots tips. All of these cells are characterized by a lack of functional plasmodesmata, which blocks the acquisition of nutrients by a symplastic route. *COPT 1* antisense plants have decreased Cu levels as a result of

decreased Cu uptake and show sensitivity to Cu chelators. These plants have also a pollen-development defect and root-elongation phenotype, both of which are reversed by Cu feeding. Thus, its participation in root elongation, pollen development and apoplastic Cu transport has been proposed (Sancenón et al. 2004). COPT1 plays an important physiological role in root Cu acquisition and accumulation since it is required for growth under Cu limiting conditions.

Subsequent members of COPT family have been identified by sequence homology to COPT1 and yeast (*Saccharomyces cerevisiae*) complementation (Sancenón et al. 2003). The existence of three COPT groups according to the number of *N*-terminus methionine- and histidine- rich boxes has been proposed. The first one, including COPT1 and COPT2, displays the more high-affinity Cu transporter features being probably plasma membrane proteins. The second group includes the COPT3 and COPT5 transporters having only one methionine- and histidine- rich box, which shows partially level of both complementation and Cu transport rate. COPT3 and COPT5 probably participate in intracellular Cu transport. Putative target sequences to the chloroplast and the secretory pathway have been predicted for COPT3 and COPT5, respectively. COPT4 represents a third group showing high level expression in roots that lacks methionine residues and motifs essential for Ctr1-mediated high-affinity Cu transport. These findings suggest a non-direct role in Cu transport (Sancenón et al. 2004) and its function in Cu homeostasis is currently questioned. An additional member of COPT family, named COPT6, has been identified recently. Further characterization will be necessary to know its putative role in Cu transport.

### 2.3.2 P<sub>1B</sub>-type ATPase transporters

P-type heavy metal ATPases are involved in the transport of a range essential and potentially toxic metals (*i.e.*, Cu<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup>) across cell membranes (Solioz and Vulpe 1996; Palmgren and Axelsen 1998). They transport metals across membranes following the classical E1/E2 Albers-Post catalytical cycle (Kühlbrandt 2004, Argüello et al. 2007). Sequence comparisons generally

group  $P_{1B}$ -type ATPases into two further classes: *i*) those transporting monovalent cations as Cu/Ag and *ii*) those transporting divalent cations as Cd/Pb/Zn/Co (Axelsen and Palmgren 2001; Cobbet et al. 2003). Structurally,  $P_{1B}$ -type ATPases contain eight transmembrane (TM) segments with various cytoplasmic domains involved in enzyme phosphorylation (P-domain), nucleotide binding (N-domain) and energy transduction (A-domain), domains that are common for all P-type ATPases (Fig. 2). Additionally,  $P_{1B}$ -ATPases show different features associated with their singular function in heavy metal transport such as *i*) metal transmembrane binding sites responsible for metal recognition and movement across the membrane permeability barrier, and *ii*) N- and C-termini metal binding domains with highly conserved CxxC motif that control the enzyme turnover rate without affecting metal binding to transmembrane transport sites (Argüello 2003; Argüello et al. 2007). The mechanism operating during metal delivery to metal transmembrane binding sites is still not clear but the requirement of conserved amino acid residues in the transmembrane region has been proposed (Argüello 2003; Argüello et al. 2007). More recently, the structure of two transmembrane transport sites with high metal affinity has been determined in a  $P_{1B}$ -ATPase transporting  $Cu^+$  (Argüello et al. 2008). Site I constituted by two cysteines in TM6 (CPC motif) and a tyrosine in TM7 and site II formed by asparagine in TM7 and methionine and serine in TM8. Both sites can be independently loaded with  $Cu^+$  but their simultaneous occupation is associated with enzyme turnover. It has been postulated that chaperone can deliver  $Cu^+$  directly to the transmembrane metal-binding sites, suggesting that in this model the N-terminus metal binding-site has a regulatory function without participating in metal transport (González-Guerrero and Argüello 2008; Chen-Chou 2008).

Plants differ significantly from other organisms in the number and selectivity of their  $P_{1B}$ -ATPases (Williams and Mills 2005). For instance, the number of genes encoding  $P_{1B}$ -ATPases can vary among species. *Arabidopsis* genome encodes eight members of the  $P_{1B}$ -type ATPase subfamily,

also known as HMA transporters (*AtHMA1-AtHMA8*), a number similar to other non-plant eukaryotic species, which have been characterized to some extent. These proteins differ in their structure, function and regulation but all of them are specialized in specific metal ion transport to cellular compartments and targets proteins (Baxter et al. 2003; Williams and Mills 2005). The rice (*Oryza sativa* L.) genome contains nine  $P_{1B}$ -type ATPase genes and ten members of this subfamily have been identified in barley (*Hordeum vulgare* L.).

*AtHMA1* to *AtHMA4* belong to the group implicated in divalent cations transport, and *AtHMA5* to *AtHMA8* act in transport of monovalent  $Cu^+$  ions. Based on their amino acid sequences and topological arrangements, and combining this with their metal affinity the  $P_{1B}$ -ATPases (HMAs) have been classified into six subgroups ( $P_{1B-1}$  -  $P_{1B-6}$ ) (Argüello 2003; Argüello et al. 2007). The first member cloned in plants was PAA1 (*AtHMA6*) ( $P_{1B}$ -type ATPase of *Arabidopsis* 1) from *Arabidopsis thaliana* L. (Tabata et al. 1997), which shows similarity to the cyanobacterial CtaA protein. Later, Shikanai et al. (2003) demonstrated that PAA1 (*AtHMA6*) is responsible for the delivery of Cu to chloroplasts, which provides the cofactor for the stromal Cu/ZnSOD enzyme and for the thylakoid lumen protein plastocyanin. *paa1* mutants have a high chlorophyll fluorescence phenotype arising from impaired photosynthetic electron transport apparently because of a deficiency in holoplastocyanin. The phenotype can be rescued by the addition of excess Cu to the growth medium. PAA2 (*AtHMA8*), closely related to PAA1 (*AtHMA6*), shows similarity to PacS transporter from cyanobacteria and transports Cu into the thylakoid lumen to supply plastocyanin (Abdel-Ghany et al. 2005b). A double *paa1paa2* mutant resulted in seedling lethality, a more severe phenotype than that observed for plants defective for both genes, underlying the importance of Cu to photosynthesis (Weigel et al. 2003; Abdel-Ghany et al. 2005b). The phenotypes of *paa1* and *paa2* mutants were reverted by addition of exogenous Cu but not of the *paa1paa2* double mutant, suggesting that an alternative lower-affinity pathway for Cu delivery can exist in chloroplasts.

Recently, the homolog of PAA2 (*AtHMA8*) in soybean (*Glycine max* L. var. Corsoy) named *GmHMA8* has been identified and localized in the thylakoid membrane (Bernal et al. 2007b).

Proteomic analyses of the *Arabidopsis* chloroplast envelope identified *AtHMA1* as a new candidate for the alternative Cu transport into this organelle (Seigneurin-Berny et al. 2006). *AtHMA1* localizes in chloroplast envelope and affects Zn- and Cu-uptake activity when expressed in yeast. It is worth mentioning that the *AtHMA1* protein does not contain the MxCxxM N-terminus Cu<sup>+</sup>-binding motifs (Fig. 2), but instead, it is histidine rich at the N-terminus domain, suggesting that it may transport Cu<sup>2+</sup> rather than Cu<sup>+</sup>. Characterization of *hma1* mutants revealed lower Cu content in chloroplasts and a reduction of the chloroplast Cu/ZnSOD activity, but normal plastocyanin content, suggesting that *AtHMA1* could deliver divalent ions including Cu<sup>2+</sup> and Zn<sup>2+</sup> to Cu/ZnSOD in plastids. The idea that Cu<sup>2+</sup> may be present in the space between both chloroplast envelopes is supported by the existence of the chloroplast Cu<sup>2+</sup>-binding protein *AtCutA* (Burkhead et al. 2003) in this location. However, the responsible transporters for this alternative transport activity in thylakoids have not been described yet. Furthermore, *AtHMA1* may have specific functions in plants grown under adverse light conditions (Seigneurin-Berny et al. 2006).

The Responsive to Antagonist (RAN1)/*AtHMA7* was identified in a genetic screen for plants with an unusual response to the ethylene antagonist *trans*-cyclooctene, underscoring the critical role of Cu in the ethylene-signalling pathway (Hirayama et al. 1999). This role is explained by the fact that ethylene receptors are Cu-dependent proteins (Rodríguez et al. 1999; Hirayama and Alonso 2000). In *Arabidopsis*, RAN1 (*AtHMA7*) was the first functionally characterized heavy metal ATPase. RAN1 (*AtHMA7*) is involved in ethylene signalling by supplying Cu at the endoplasmic reticulum, where it is required for the formation of functional ethylene receptors (Woeste and Kieber 2000; Chen et al. 2002). The plant hormone ethylene is an important signal in many abiotic stress situations but also in plant pathogen interaction. RAN1 (*AtHMA7*) has also been found in rapeseed

(*Brassica napus*), *BnRAN1*, (Southron et al., 2004). Among the rice P<sub>1B</sub>-ATPases, *OsHMA9* was found to form a subclass with *RAN1* (*AtHMA7*). The recent characterization of *OsHMA9* indicated that it plays a role in Cu detoxification acting as an efflux pump in the plasma membrane (Sichul et al. 2007). Mutant *oshma9-1* and *oshma9-2* plants exhibited the phenotype of increased sensitivity to high levels of Cu, and also Zn and Pb. The *OsHMA9* gene was mainly expressed in vascular tissues, including xylem and phloem and weakly expressed in mesophyll tissues. In developing tissues, expression was strong in anthers, suggesting a putative role in metal delivery to rice anthers. The importance of metal transport in anthers has been previously reported.

The *Arabidopsis AtHMA5*, the closest homolog of *RAN1* (*AtHMA7*) in the P<sub>1B</sub>-type ATPase subfamily, is strongly and specifically induced by Cu in whole plants (Fig. 2). The *hma5* T-DNA insertion mutants are hypersensitive to Cu and HMA5-defective plants accumulate Cu in roots to a greater extent than wild-type plants, suggesting its key role in transmembrane transport, and particularly in root Cu detoxification (Andrés-Colás et al. 2006). This phenotype is the opposite of that observed for the COPT antisense lines, supporting the notion that COPT1 and *AtHMA5* transport Cu in opposite directions. *AtHMA5* is mostly expressed in roots, flowers and pollen. The specific interaction of *AtHMA5* with two different ATX1-type chaperones, ATX1 and CCH, in *Arabidopsis thaliana* has been demonstrated. Although further experiments are necessary to confirm the fact, it has been proposed that *AtHMA5* could be involved in Cu efflux at specific root cells and its overexpression in plants could be a strategy for improving Cu detoxification under Cu excess.

### 2.3.3 Copper chaperones

The Cu chaperones belong to a new family of cytosolic, soluble, low-molecular-weight metal-receptors proteins named metallochaperones that are involved in the intracellular trafficking of metal ions and insert the Cu into the active sites of specific partners, Cu-dependent enzymes (O'Halloran

and Culotta 2000; Huffman and O'Halloran 2001). The limited solubility and high reactivity of Cu<sup>+</sup> inside the cell requires the participation of these specialized proteins that prevent inappropriate Cu interaction with other cellular components. Cu chaperones are conserved in most eukaryotes, but specific characteristics seem to emerge in plants. In yeast, the P-type ATPase transporter named Ccc2p interacts with a small cytosolic Cu chaperone named Antioxidant1p (Atx1), which delivers Cu to the Ccc2p by direct protein-protein interaction. *Arabidopsis* has two homologs of the yeast ATX1 chaperone named Copper Chaperone (CCH) and ATX1 (Himmelblau et al. 1998; Andrés-Colás et al. 2006). CCH has been the most extensively studied of the Cu chaperones in plants (Mira et al. 2001a, b). The CCH chaperone exhibits the conserved features of the ATX1-type metallochaperone family such as typical lysine residues, overall  $\beta\alpha\beta\beta\alpha\beta$  fold structure and an MxCxxC Cu<sup>+</sup>-binding motif in the N-terminus (Pufahl et al. 1997). However, CCH also presents a plant-exclusive C-terminal domain with special structural characteristics (Mira et al. 2001a,b; Mira et al. 2004) that makes CCH unique and distinct from the non-plant ATX1-type chaperones. CCH-like C-terminus domains have been only found in higher plants, suggesting a regulatory role for that. Both the CCH and ATX1 chaperones complement the yeast *atx1* mutant and interact with the N-terminus of *AtHMA5* (Andrés-Colás et al. 2006). However, the C-terminus of CCH has a negative effect on its interaction with *AtHMA5*. The plant *CCH* gene expression has been related to oxidative stress and senescence, when the plant reallocates nutrient resources. High levels of *CCH* expression were found in *Arabidopsis* stems and vascular cells that lack nuclei. A plant-specific role in Cu symplastic transport through the plasmodesmata during senescence associated with nutrient mobilization has been proposed for this extra C-terminus domain of CCH. Expression of *CCH* increases by oxidative stress, senescence, and Cu deficiency. A CCH chaperone has been also identified by differential display in tomato (*LeCCH*) infected with the fungal pathogen *Botrytis cinerea* (Company and González-Bosch 2003) suggesting an interesting relationship between Cu homeostasis and plant defence responses.

The COX17 chaperone shares sequence similarity to COX17 from yeast that might mediate the delivery of Cu to the mitochondria for the assembly of a functional cytochrome-c oxidase complex (Balandin and Castresana 2002). In this manner COX17 would contribute to the increase in activity of specific enzymes that are required to preserve organelle functionality in a number of biotic and abiotic stress situations.

Despite their role in Cu homeostasis, neither CCH nor RAN1 (*AtHMA7*) are induced by Cu treatment, indicating that they might be more important in helping cells cope with Cu deficit than Cu excess. In contrast, activation of *AtCOX17* gene expression in response to Cu treatment might be an indication of a function like metallothioneins, which are also induced by high concentrations of metals (Zhou and Goldsbrough 1995). Nevertheless, further experimental support is necessary to establish the function of these proteins.

The *CCS* gene, homolog of the yeast *Ccs1p/Lys7p* gene, encoded a protein that delivers Cu to the Cu/ZnSOD by a protein-protein interaction. It has been identified in tomato (*LeCCS*) (Zhu et al. 2000), *Arabidopsis thaliana* (Wintz and Vulpe 2002), potato (*Solanum tuberosum* L.; *StCCS*) (Trindade et al. 2003), maize (*ZmCCS*) (Ruzsa and Scandalios 2003) and soybean (*GmCCS*) (Sagasti S, Bernal M, Picorel R, Yruela I, unpublished results). *AtCCS* has a predicted chloroplast targeting sequence but dual localization in both cytosol and plastids (Chu et al. 2005). Therefore is possible that *AtCCS* delivers Cu to both cytosolic and chloroplastic Cu/ZnSOD enzymes, perhaps using an alternative translation start site. It has been shown that *AtCCS* is Cu up-regulated and co-regulated with cytosolic and chloroplastic Cu/ZnSOD targets indicating an important role in the regulation of oxidative stress protection. An up-regulation of *AtCCS* mRNA has been also found in response to senescence. Additionally, *AtCCS*, and both cytosolic and chloroplastic Cu/ZnSODs were down-regulated in response to Cu deficiency. It has been also proposed that *AtCCS* expression is regulated to allow the most optimal use of Cu for photosynthesis (Abdel-Ghany et al. 2005a).

*StCCS* gene expression was induced by auxin which is known to play a role in different



stages of potato (*Solanum tuberosum*) development. Auxins have a promoting effect on cell elongation/expansion. Surprisingly, potato (*Solanum tuberosum* L.) plants sprayed with  $\text{CuSO}_4$  did not respond with a significant change in *StCCS* expression (Trindade et al. 2003). This is consistent with the inhibition of *StCCS* gene expression observed when potato plants were grown *in vitro* in media supplemented with 10 mM  $\text{CuSO}_4$ . This surprised finding may be explained if the presence of a chaperone would not be required for the incorporation of Cu in the Cu/ZnSOD when Cu is present at high concentrations in leaves.

#### 2.3.4 ZIP transporters

ZIP proteins belong to divalent metal transporters family and generally contribute to metal ion homeostasis through the transport of cations into the cytoplasm (Colangelo and Guerinot 2006; Puig et al. 2007). They contain eight transmembrane (TM) domains and a histidine-rich variable loop between TM3 and TM4. IRT1 (Iron-Regulated Transporter 1) is the best characterized member of ZIP family in plants. Fe acquisition in *Arabidopsis* roots under Fe deficiency mostly depends on *AtIRT1*, which is considered the major Fe transporter at the root surface in *Arabidopsis thaliana*. The closely *OsIRT1* appears to play similar role in Fe uptake under Fe limiting conditions in rice. The ZIP family contains 14 additional members in *Arabidopsis* (Mäser et al. 2001). *AtZIP2* and *AtZIP4* complement growth defects of yeast Cu and Zn transport mutants (Grotz et al. 1998; Wintz et al. 2003). Expression of both genes is up-regulated in *Arabidopsis* by deficiency in Cu and Zn, but not in Fe. It has been proposed that *AtZIP2* participates in Cu acquisition by *Arabidopsis* roots. Although the role of these proteins in plant Cu transport still requires further characterization, the preference that ZIP family members show for divalent metals suggest that ZIP2 and ZIP4 proteins may transport  $\text{Cu}^{2+}$  ions.

Six cDNA encoding ZIP family members have been identified in the model legume

*Medicago truncatula* L. and tested for the ability to complement yeast metal-uptake mutants (López-Millán et al. 2004). A role in metal homeostasis has been proposed based on expression analysis of mRNA levels in response to metal supply.

### 2.3.5 Nramp transporters

Nramp family members are implicated in the transport of several divalent metal ions. In plants, investigations of Nramp family were largely restricted to rice (*Oryza sativa*) where three members were identified, *OsNramp1*, *OsNramp2* and a partial length *OsNramp3* (Belouchi et al. 1995; 1997). Subsequently, two *Arabidopsis* genes were identified (Alonso et al. 1999) which showed similarity to *Nramps*. More recently, three additional genomic sequences from *Arabidopsis* with homology to *Nramps* have been found named *AtNramp1*, *AtNramp3* and *AtNramp4*. Comparisons of pair wise similarities between each of these genes suggests that the plant *Nramps* can be broadly divided into two groups: 1) *OsNramp1*, *OsNramp3* and *AtNramp5* which share high similarity and 2) *OsNramp2*, *AtNramp1*, *AtNramp2*, *AtNramp3* and *AtNramp4*, which have lower similarity to group (1). This finding could suggest the possibility of subgroups that may vary in their substrate specificity, although this remains to be demonstrated. As with other members of this family, the plant Nramp proteins have twelve predicted transmembrane domains, however, it also possesses a long intracellular C-terminus tail which is unique to the Nramp proteins. A transport function for the plant Nramp homologues remains to be formally demonstrated; however there is good evidence from yeast studies for a role of the Nramp proteins in divalent cation transport. In *Arabidopsis* Nramp1 (*AtNramp1*) confers tolerance to toxic concentrations of external Fe (Curie et al. 2000). Homologues of *Nramp* family have been also identified in soybean proposing to be involved in Fe<sup>2+</sup> transport and Fe homeostasis in the nodule to support symbiotic N<sub>2</sub> fixation (Kaiser et al. 2003). However, they have also been shown to be mediating the uptake to other metal ion such as Cu in yeast. Therefore a similar function in plants should be not dismissed.

### 2.3.6 Mugineic acid and nicotianamine.

Since very little metal in plants is assumed to exist as free ions, a number of small organic molecules have to be implicated in metal ion homeostasis as metal ion ligands or chelators in order to improve acquisition and transport of metal ions with low solubility, and immobilization for metal tolerance and storage. Among these ligands mugineic acid (MA) and nicotianamine (NA) have been shown to participate in the transport of essential metals such as Cu, Fe, Mn, Ni or Zn. Nicotianamine (NA), which is a precursor of mugineic acid (MA), is an ubiquitous metal-chelator in all plants and, like MA, is believed to play a primary role in metal homeostasis (Haydon and Cobbet 2007). *In vitro*, NA is able to form stable complexes with Mn, Fe, Co, Zn, Ni and Cu, in increasing order of affinity (Curie et al. 2009). The stability of all metal-NA complexes is maximal at pH 6.5 indicating that NA would be more likely a symplastic chelator of metals but among essential metals Cu is the exception as the Cu-NA complex being very stable in mild acidic conditions. This fact favours the possible occurrence of Cu-NA complex in an apoplastic environment such as the xylem. NA is synthesized by nicotianamine synthase (NAS) from S-adenosyl-L-methionine. The first evidence for a role of NA in metal transport came from Cu- and Fe- related phenotypes associated with the NA synthesis-defective *chloronerva* tomato mutant, which showed interveinal chlorosis (Ling et al. 1996; Mori 1999). Later, studies in NA-defective tobacco (*Nicotiana sp.*) plants pointed to the essentiality of NA for metal transport in veins and interveinal areas, and for reproductive growth and fertility.

*Nicotianamine synthase (NAS)* genes were up-regulated in roots and shoots of plants grown under Cu, Fe or Zn deficiency. Recent evidence for the role of NA in plants comes from depletion of NA in tobacco (*Nicotiana sp.*) by transgenic overexpression of *Nicotianamine aminotransferase (NAAT)* from barley. The levels of Cu, Fe and Zn decreased in leaves and floral organs of transgenic plants, suggesting a role for NA in long-distance translocation of these metals. In a reciprocal experiment, overexpression of barley *NAS* in transgenic tobacco (*Nicotiana sp.*) lead to increased Cu, Fe and Zn content in leaves and flowers and enhanced the Fe and Zn content of pollen and seeds, further

supporting a role for NA in transport of these metals (Takahashi et al. 2003). This could be consistent with NA should complex Cu, Fe and Zn in the phloem and Cu and Zn in the xylem for their translocation from roots to shoots (von Wiren et al. 1999). The finding that Cu-NA complex is completely stable at the pH of xylem sap (pH 5-6) supports this assumption (Curie et al. 2009).

### 2.3.7 YSL transporters

The Yellow Stripe-Like (YSL) transporters belong to the oligopeptide transporter (OPT) superfamily (Curie et al. 2001; Curie et al. 2009), which transport tri-, tetra-, penta- and hexapeptides (Yen et al. 2001). YSL proteins are also believed to mediate the uptake of metals that are complexed with plant-derived phytosiderophores (PS) or nicotianamine (NA) (Colangelo and Guerinot 2006). Thus, the assumption that the members of the OPT family transport only peptides is being challenged since some OPT protein may also be capable of divalent metal ions transport. The best-studied member of this family is YS1 from maize (Roberts et al. 2004; Schaaf et al. 2004). *ZmYS1* protein accumulates in roots and leaves of Fe-deficient plants and functions as a proton-coupled symporter to transport Fe-PS and may also play a role in the homeostasis of Cu, Zn or Ni as mugineic (MA)-complexes. On the basis of sequence similarity to *ZmYS1*, *A. thaliana* has eight predicted YSL proteins.

Considering that non-grasses plants do not produce or use PS, *AtYSL* proteins most probably transport metal-NA complexes. Two family members, *AtYSL1* and *AtYSL2*, have recently been studied in some detail. *AtYSL2* transcript accumulation increases under conditions of Fe sufficiency or Fe resupply, and *AtYSL2* transcript levels also respond to Cu and Zn (DiDonato et al. 2004; Schaaf et al. 2005). The expression of *AtYSL2* in metal-uptake-defective yeast strains mediated the uptake of Fe-NA and Cu-NA. Localization of *Arabidopsis* YSL2 in root endodermis and pericycle cells facing the meta-xylem tubes has suggested its participation in lateral movement of Fe and/or Cu within the veins (Schaaf et al. 2005). These proteins seem to be involved in the unloading of metal-NA from vasculature into developing tissues, in immobilization of metal-NA from senescent leaves and in an

efficient loading of metal-NA into seeds.

*AtYSL1* transcript levels increase in response to high Fe conditions (Le Jean et al. 2005). *Arabidopsis YSL1*-defective mutants contain lower levels of Fe-NA in their seeds and display a transient defect in germination that can be rescued by Fe supply. It has been shown that *AtYSL1* and *AtYSL3* were up-regulated during leaf senescence. The *ysl1ysl3* double knockout mutants, which exhibit interveinal chlorosis in leaves caused by decreased Fe levels and reduced fertility as a consequence of defective anther and embryo development were less efficient in mobilizing metals, especially Cu, from senescent leaves. These results and *YSL1/YSL3* expression in the vasculature of shoots and reproductive organs suggest a function in Cu delivery among other metals from vascular tissues, as well as in Fe-NA delivery to seeds (Waters et al. 2006). *AtYSL2* and *AtYSL3* are differentially expressed under metal deficiencies, and heterologous expression of *AtOPT3* in yeast suggests that it can transport  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Fe}^{2+}$  (Wintz et al. 2003).

The rice (*Oryza sativa*) genome contains 19 putative *OsYSL* genes. *OsYSL2* has been shown to transport  $\text{Fe}^{2+}$ -NA and  $\text{Mn}^{2+}$ -NA complexes but not  $\text{Fe}^{3+}$ -NA. A role in the transport of divalent cations in the phloem has been suggested (Koike et al. 2004; Colangelo and Gueriot 2006). Current investigations point out the role of YSL proteins in long-distance metal-NA chelate transport and development of pollen grains and seeds (Curie et al. 2009) but further studies are necessary to clarify if specific members of YSL family have substrate specificity.

## 2.4 Regulatory mechanisms

In plants, the regulatory mechanism of gene expression is a relatively new area of research. Particularly, there are still little indications of how genes encoding metal transporters are regulated in higher plants. This could occur potentially at the transcriptional level (control on initiation rates, differential mRNA splicing, mRNA stability) or at the post-translational level (targeting, stability). Many metal transporters in other organisms are regulated at the transcriptional level by extracellular

metal concentrations *via* transcription factor proteins (Radisky 1999). Studies in model organisms such as the green alga *Chlamydomonas reinhardtii* and the yeast *Saccharomyces cerevisiae* outlined a number of principles of metal regulation. Metal-sensing transcription factors controlling the transcription of target genes are a common feature in metal acquisition. These are examples for very direct metal-dependent regulation, not requiring upstream signal transduction cascades. For instance, under Cu deficiency the Cu-binding Mac 1p (metal-binding activator 1) transcription factor of *S. cerevisiae* binds as a homodimer to copper-responsive sequence elements (CuRE) in the promoters of the genes *ScCtr1* and *ScCtr3*, which encode Cu<sup>+</sup> uptake systems (Labbe et al. 1997; Zhu et al. 1998; Rutherford and Bird 2004; Krämer and Clemens 2006). Mac 1p is able to bind four Cu<sup>+</sup> ions in a poly-copper cluster within its transactivation domain. The binding of Cu<sup>+</sup> to Mac 1p triggers an interaction between the transactivation and DNA binding domains of Mac 1p, inhibiting the functions of both domains. Under conditions of Cu excess, the Cu-dependent transcriptional activator *ScAce1p* is activated by the binding of four Cu<sup>+</sup> ions and activates the transcription of genes involved in the protection of yeast cells from Cu toxicity, such as the gene encoding the Cu-buffering cysteine-rich Cup1 protein (Rutherford and Bird 2004).

In plants, depending on Cu status, a mechanism of Cu-responsive transcriptional repression *via* SBP-related transcription factors (SPL) that bind to GTAC sequences within the promoter region regulates the replacement of chloroplastic FeSOD by Cu/ZnSOD in response to Cu by directly repressing the transcription of the *FeSOD* gene and indirectly inducing the transcription of the *CuZnSOD* (Nagae et al. 2008).

Although numerous animal and human genes are alternatively spliced (Green 1991), the role of this type of regulatory mechanism of gene expression in plants is a relatively new area of research (Kazan 2003). The great majority of alternatively spliced genes in *Arabidopsis thaliana* encode proteins with regulatory functions. Additionally, genes associated with various stress (biotic, water,

light, salt, wounding, heavy metal, heat) responses seem to be particularly prone to alternative splicing in both animals and plants (Kazan 2003). Among those, *AtCutA* mRNA that encodes a chloroplast protein involved in Cu tolerance in *Arabidopsis* is regulated by alternative splicing (Burkhead et al. 2003).  $P_{1B}$ -ATPases seem to be also alternatively spliced, at least in certain plants. Bernal (2006a) demonstrated that *GmHMA8*, a member of the soybean  $P_{1B}$ -ATPases subfamily, is subject of alternative splicing, whereby retention of an intron yield a non-spliced (NSP) transcript named NSP-*GmHMA8*. The putative non-spliced NSP-*GmHMA8* protein contains six transmembrane (TM) domains, two TMs shorter than typical  $P_{1B}$ -ATPases including *GmHMA8*. In humans, there is evidence that splicing regulates both the Menkes (ATP7A/MNK) and the Wilson (ATP7B/WMN)  $Cu^+$ - $P_{1B}$ -ATPases highly homologues to *GmHMA8*. Generally, many spliced products in humans show tissue-specific expression (Lutsenko et al. 2007).

Another important level of metal regulation is the metal-dependent regulation of transcript stability. Cu-dependent transcriptional regulation has been shown in the unicellular green alga *Chlamydomonas reinhardtii*. Apoplastocyanin is rapidly degraded when Cu is not available for the formation of holoplastocyanin, probably through a non-specific pathway (Merchant and Bogorad 1986a,b). Thus, the availability and insertion of the metal cofactor into apoplastocyanin controls the stability of the translation product in the chloroplast of *C. reinhardtii*. Although not all apometalloproteins are unstable, the insertion of metal ion cofactors is likely to be an important factor controlling the activity and/or stability of proteins, and possibly of biological processes (Krämer and Clemens 2006).

Additionally, it has been shown that Cu regulates the expression of certain members of  $P_{1B}$ -ATPases, COPT transporters, and Cu chaperones. For instance, the expression of *AtHMA5* in *Arabidopsis*, and *OsHMA5* and *OsHMA9* in rice,  $P_{1B}$ -ATPases involved in Cu detoxification, is stimulated by excess Cu at the transcriptional level (Andrés-Colás et al. 2006; Sichul et al. 2007). On

the contrary, excess Cu reduces the transcript level of PAA2 (*AtHMA8*) and *GmHMA8* (Schiavon et al. 2007; Bernal 2006a). *COPT1* mRNA levels increase when Cu is limited (Sancenón et al. 2003). Concerning Cu chaperones, *AtCOX17* expression was up-regulated and *AtATX1* expression was down-regulated in response to excess Cu supply (Baladin and Castresana 2002; Schiavon et al. 2007). By contrast, *CCS* expression was not significantly influenced by Cu in *Arabidopsis* (Schiavon et al. 2007) whereas the accumulation of *CCS* mRNA was strongly increased in soybean (Sagasti S, Bernal M, Picorel R, Yruela I, unpublished results).

In higher plants, there is little evidence for the post-transcriptional regulation of metal homeostasis proteins. However, there is solid evidence for the regulation of sub-cellular protein localization and stability in yeast and humans. Thus, it is likely that similar mechanisms operate in higher plants. An example of this post-transcriptional mechanism of metal regulation is the metal-dependent re-localization or degradation of metal transport proteins. The ATP7A/MNK P<sub>1B</sub>-ATPase responsible for Menkes disease in humans, and highly homologue to PAA2 (*AtHMA8*) and *GmHMA8* transporters in plants, exhibits a Cu-dependent subcellular localization. The protein was proposed to cycle continuously between the Golgi and the plasma membrane. Under most conditions the major proportion of the ATP7A/MNK protein localizes predominantly to the *trans*-Golgi, supplying Cu to the lumen of this compartment. Under exposure to high Cu concentrations, localization is shifted towards the plasma membrane, where the bulk of this transporter exports Cu to the exterior of the cell. Furthermore, in Cu-deficient cells, the human Cu uptake transporter *hCtr1*, highly homologue to *COPT1* in plants, localizes to the plasma membrane, but undergoes Cu-stimulated endocytosis under Cu resupply. Two putative Cu-binding methionine-rich sequence elements of *hCtr1* are involved in the regulation of endocytosis, suggesting that direct Cu sensing by *hCtr1* may be controlling its localization (Guo et al. 2004).

Plants can be postulated to contain specific metal sensors that detect changes in metal status (deficiency or excess) and trigger signalling cascades that activate the appropriate responses. In



higher plants, the signal transduction pathways involved have not been identified yet. Jonak et al. (2004) observed that toxic concentrations of Cu activated mitogen-activated protein kinases (MAPKs) in *Medicago sativa* seedlings, suggesting that MAPK pathways are activated in response to excess Cu. MAPKs are involved in signal transduction induced by heavy metals and protein phosphorylation events. It remains to be established to which extent the activation of the respective MAP kinase cascades is metal-dependent or an effect of oxidative stress.

Recently, several exciting findings have revealed the regulation of micro-RNAs (miRNAs) expression by specific nutrient stresses (Chiou 2007). The novel function for miRNAs in regulating plant adaptive responses to nutrient stresses opens up an interesting field to research. The role of miR398 in the expression patterns of *CSD1* and *CSD2* mRNAs has been reported (Sunkar et al. 2006). Particularly, they show that miR398 expression is downregulated transcriptionally by oxidative stresses, and this downregulation is important for posttranscriptional *CSD1* and *CSD2* mRNA accumulation and oxidative stress tolerance. Transgenic *A. thaliana* plants overexpressing a miR398-resistant form of *CSD2* accumulate more *CSD2* mRNA than plants overexpressing a regular *CSD2* and are consequently much more tolerant to high light, heavy metals, and other oxidative stresses. Evidence that several miRNA families mediate the regulation of Cu-containing proteins in *A. thaliana* in response to Cu status has been recently reported (Yamasaki et al. 2007, Abdel-Ghany and Pilon 2008). The transcription of *miR398* is repressed by Cu and *miR398* mediates down-regulation of chloroplastic Cu/ZnSOD (*CSD2*) in *A. thaliana* in response to changes in a low range of Cu levels (0.2-0.5  $\mu$ M) (Yamasaki et al. 2007), indicating that *miR398* is rather involved in a response to Cu limitation. A multiple copies of GTAC sequences were found in *miR398* promoter sequences suggesting that Cu promotes the expression of the chloroplastic *Cu/ZnSOD* (*CSD2*) posttranscriptionally by repressing the transcription of *miR398* through GTAC sequences motif (Nagae et al. 2008). This GTAC sequence-dependent transcriptional regulatory mechanism by Cu seems to be conserved in land plants. The down-regulation of chloroplastic Cu/ZnSOD (*CSD2*) on

low Cu would contribute to maintaining a Cu pool for plastocyanin allowing plants to save Cu for essential functions such as photosynthetic electron transport (Yamasaki et al. 2007). More recently, Abdel-Ghany and Pilon (2008) have found that *miR397*, *miR408* and *miR857* together regulate other Cu-containing proteins such as plantacyanin, and a number of laccases. These authors have proposed that Cu related miRNAs are used in response to avoid Cu deficiency since they are up-regulated already in a condition where symptoms of deficiency are still absent and where plastocyanin function is not compromised.

#### 2.4.1 Responses to copper deficiency

Cu can be limiting to plant productivity when below  $5 \mu\text{g g}^{-1}$  dry weight. Cu-deficient plants show changes in the expression of a series of genes and activation of morphological changes either in root or leaf architecture. Typical symptoms of Cu deficiency appear first at the tips of young leaves and then extend downward along the leaf margins. The leaves may also be twisted or malformed and show chlorosis (*i.e.*, loss of chlorophyll) or even necrosis; the overall biomass of affected plants is subsequently reduced. These symptoms are known for a long time (for review see Marschner 1995; Küpper and Kroneck 2005) and can be explained in view of the roles of Cu in plant metabolism. Thus, the lack of Cu reduces PSI electron transport due to decreased formation of plastocyanin (Baszynski et al. 1978; Shikanai et al. 2003), which is the major target of Cu deficiency in photosynthesis. Decrease in PSII activity was also observed in Cu-deficient chloroplasts (Droppa et al. 1987; Henriques 1989). Droppa et al. (1987) concluded that severe Cu deficiency changes the thylakoid membranes and modifies the ambient of the PSII acceptor side. They also noticed the absence of a 29 kDa polypeptide, which is probably a component of CP29, a minor chlorophyll a/b binding protein of PSII. Cu-deficient plants show disintegration of the thylakoid membranes of chloroplasts (Baszynski et al. 1978; Henriques 1989) as well as decreased pigment (chlorophylls and carotenoids) content, reduced plastoquinone synthesis and lower unsaturated C18 fatty acids contents

(Barón et al. 1992). Availability of Cu also affects Cu/ZnSOD enzyme diminishing their expression and activity.

Current knowledge establishes that at least three different molecular strategies can be distinguished in response to Cu deficiency in plants. The first one is conducted to improve metal acquisition and includes increased expression of metal reductases and high-affinity transporters. The second one consists in prioritizing the use of metals in essential *versus* non-essential pathways. Finally, if metalloproteins with different metallic ligands perform similar or overlapping functions, a specific metalloprotein can be substituted by another when its metal is deficient (Puig et al. 2007). The up- and down- regulation of genes directing the events mentioned above involve a series of molecular mechanisms that begin with the plant “sensing” the deficiency and then transmitting the signal along transduction pathways through the plant vascular system. Signals between the aerial parts of the plants, including the apical meristem, and the roots lead to the activation or inactivation of transcription factors that influence expression of specific genes. Thus, plants respond to a change in metal supply by marked alterations in their transcriptome. Genetic and biochemical studies in model organisms (*i.e.*, the green alga *C. reinhardtii*) have established that transcriptional regulation control is the primary response to Cu deficiency. The up-regulation of genes in response to Cu limitation in *Chlamydomonas* (*i.e.*, *cytochrome c<sub>6</sub>*, *Cyt c<sub>6</sub>*, and *coproporphyrinogen III oxidase*, *CPXI*) is dependent on Cu-responsive elements (CuREs) in the 5′ upstream region of Cu-deficiency induced genes, with critical GTAC core sequences as responsible for transcription activation of genes under deficiency conditions (Quinn et al. 2000; 2002). Based on a genetic screen, Eriksson et al. (2004) revealed that the Copper Response Regulator 1 (*Crr1*) is responsible for *Cyt c<sub>6</sub>* and *CPXI* activation upon Cu limitation. The Crr1 protein shares some similarity with the plant DNA-binding domain named squamosa-promoter- binding-protein family (SBP) and contains Zn fingers in its DNA-binding domain (Kropat et al. 2005). The Crr1-SBP domain specifically binds CuRE within

*Cyt c<sub>6</sub>* and *CPXI* promoter regions. Other interesting feature is that Crr1-protein can work as a transcriptional activator or as a repressor, depending on the position of the CuRE (Moseley et al. 2002). The *Arabidopsis* genome contains 17 proteins with a well-conserved DNA binding domain, (SBP domain), which are denoted SPL proteins (squamosa protein-like (SPL) proteins), some of them involved in flower development (Birkenbihl et al. 2005).

Cu-protein substitution by functionally equivalent Fe proteins under low Cu has been well-documented in different organisms. In plants, the chloroplastic Cu/ZnSOD (CSD2) is replaced by the FeSOD upon Cu limitation. Under this condition, chloroplastic *FeSOD* mRNA, its transcript product and the activity levels increased, while either chloroplastic or cytosolic Cu/ZnSOD levels are undetectable (Abdel-Ghany et al. 2005b). This finding is accompanied by a decrease in the expression of the corresponding Cu chaperone CCS. This coordinated regulation of nuclear encoded genes at transcriptional level is probably controlled by the optimal use of chloroplastic available Cu ions and suggest that stromal Cu levels maybe regulate nuclear expression through a still unknown signalling pathway. Recently, using transgenic moss plants (*Barbula unguiculata* L.) it was determined that GTAC motif is a negative cis-acting element of the *FeSOD* in response to Cu (Nagae et al. 2008). These authors also found that a SBP-type transcription factor (PpSBP2) and its related protein bound to the GTAC motif repressed the expression of *FeSOD*. Additionally, evidence that miRNA mediates this regulation in *A. thaliana* has been shown (Yamasaki et al. 2007, Abdel-Ghany and Pilon, 2008). More recently, it was found that the DNA binding domain of SPL7, the SPL protein most similar to Crr1 (transcription factor in *Chlamydomonas reinhardtii*), interacts with GTAC cores of the *miR398* promoter *in vitro*. SPL7 regulates the expression of *FeSOD* gene and it is involved in the switching between Cu/ZnSOD and FeSOD under Cu deficiency (Yamasaki et al. 2009). Additionally, SPL7 also activates the expression of *miR397*, *miR408* and *miR857* in low Cu conditions yielding the degradation of a series of Cu-proteins and leading to appropriate Cu

redistribution. It has been found that SPL7 activates some Cu transporters and chaperones, so it could be a master regulatory factor involved in Cu homeostasis.

Several *Arabidopsis* genes increased expression in response to low Cu availability, *i.e.*, *COPT1*, *COPT2*, *ZIP2* transporters, *FRO3* metal reductase, *CCH* chaperone and chloroplastic FeSOD (Himmelblau et al. 1998; Sancenón et al. 2003; Wintz et al. 2003; Abdel-Ghany et al. 2005b; Mukherjee et al. 2006). The theoretical analysis of the *COPT2* promoter sequence showed putative cis elements responsive to both low Fe and low Cu, suggesting that this promoter can integrate signalling pathways of deficiencies in both metals.

#### 2.4.2 Responses to copper toxicity

Toxic levels of Cu occurs naturally in some soils whereas others may contain high levels of Cu as a result of anthropogenic release of heavy metals into the environment through application of pig and poultry slurries rich in Cu, fertilizers accumulation, fungicides, industrial and urban activities, metaliferous mining or metal processing, and waste disposal technologies (Kabata-Pendias and Pendias 2001; Pilon-Smits and Pilon 2002). Cu concentration in non-contaminated soils and natural waters is *ca.* 20-30 mg kg<sup>-1</sup> and 2 µg kg<sup>-1</sup>, respectively but in contaminated soils and waters can reach levels one hundred times higher (Fernandes and Henriques 1991). Additionally, atmospheric heavy metal emission has also been identified as an important source of heavy metal contamination in plants (Friedland 1990; Salim et al. 1992). At concentrations above those required for optimal growth Cu can be toxic for most plants with the exception of a few plant species that can hyperaccumulate metals (*i.e.*, *Arabidopsis halleri* L., *Silene vulgaris* (Moench) Garcke, *Thalspi caerulescens* L.). It is worth mentioning that this toxicity is dependent on plant species, the concentration of metal supplied, exposure time and soil properties. In sensitive plant species or ecotypes Cu was shown to inhibit growth and to interfere with important cellular processes such as photosynthesis and respiration (Marschner 1995; Prasad and Strzalka 1999, Yruela 2005). In the

presence of high levels of Cu (3-100  $\mu$ M) plants normally show reduced biomass (reduction of the root and shoot volume, stem size, leaf size), chlorotic symptoms, necrosis, and inhibition of shoot and root growth. A lower content of chlorophyll and alterations of chloroplast structure and thylakoid membrane composition have been found in leaves of spinach, rice, wheat (*Triticum durum* L. cv. Adanello and Ofanto), bean (*Phaseolus coccineus* L. cv. Piekny) and oregano (*Origanum vulgare* L.) in such growth conditions (Baszynski et al. 1988; Lidon and Henriques 1991; 1993; Ciscato et al. 1997; Pätsikkä et al. 1998; Quartacci et al. 2000; Panou-Filothou et al. 2001). Particularly, degradation of grana stacking and stroma lamellae, increase in the number and size of plastoglobuli, and appearance of intrathylakoidal inclusions were observed. It has been proposed that Cu interferes with the biosynthesis of the photosynthetic machinery modifying the pigment and protein composition of photosynthetic membranes (Lidon and Henriques 1991; Maksymiec et al. 1994). Pätsikka et al. (2002) attributed the reduction of chlorophyll content to a Cu-induced Fe deficiency. The substitution of the central Mg ion of chlorophyll by Cu *in vivo* has also been proposed as a damage mechanism leading to inhibition of photosynthesis (Küpper et al. 2003; Küpper and Kroneck 2005). Besides, lipid peroxidations, decrease of lipid content and changes in fatty acid composition of thylakoid membranes were also shown (Sandmann and Böger 1980; Luna et al. 1994; Maksymiec et al. 1994). As a consequence of those modifications, an alteration of PSII membrane fluidity was found (Quartacci et al. 2000). On the other hand, the decrease of the photochemical activity caused by Cu is accompanied *in vivo* by an alteration of the structure and composition of the thylakoid membranes, which can influence the conformation and function of the photosystems (Baszynski et al. 1988, Ouzounidou et al. 1992, Lidon and Henriques 1993). Baszynski and Kruppa (1995) proposed that those processes induced by Cu could involve either the destruction of the oxygen-evolving complex polypeptide composition or the interaction with ions necessary for proper functioning of the complex as Mn, Ca and Cl.

Plant cell cultures have been widely used as suitable model system to analyse cell stress

response and adaptation, among many other studies on plant physiology. Related studies on cell culture from mesophyll cells provided information on functional cell organization changes induced by excess Cu that can be extrapolated to leaf cells in plants. Soybean cell suspensions exposed to excess Cu (10  $\mu\text{M}$ ) maintained the general cell organization pattern of the non-treated soybean cultures but excess Cu exposure induced changes in specific subcellular structures. Smaller chloroplasts with rounded shape and more numerous, with a denser structured internal membranes, no starch granules within chloroplasts and larger cytoplasmic vacuole were observed (Bernal et al. 2006b, 2006c). Similarly, chloroplasts of seven-week-old of *Arabidopsis thaliana* plants exposed to 50  $\mu\text{M}$  Cu during 2-14 days showed rather circular than ellipsoidal shape (Wójcik and Tukiendorf 2003). Starch grains disappeared and plastoglobuli became larger in chloroplasts from leaves of oregano exposed to excess Cu (10-25  $\mu\text{M g}^{-1}$ ) (Panou-Filothou et al. 2001). Roots and shoots also sense the phytotoxicity of Cu. Roots of oregano plants exposed to 13-25.5  $\mu\text{M g}^{-1}$  Cu (Panou-Filothou and Bosabalidis 2004) revealed a destroyed epidermis and a cortex of large cells with folded walls. Cortical cells exhibited a metamorphosis of the amyloplasts into leucoplasts. In root vascular cylinder, the diameter of the xylem vessels increased.

As mentioned previously, it is well known that transition metals like Cu catalyze the formation of hydroxyl radicals ( $\text{OH}^\cdot$ ) from the non-enzymatic chemical reaction between superoxide ( $\text{O}_2^{\cdot-}$ ) and  $\text{H}_2\text{O}_2$  (Haber-Weiss reaction) (Halliwell and Gutteridge, 1984). Hence, the presence of excess Cu can cause oxidative stress in plants and subsequently increase the antioxidant responses due to increased production of highly toxic oxygen free radicals. Accordingly, it was observed that excess Cu in plants led to oxidative stress inducing changes in the activity and content of some components of the antioxidative pathways (*i.e.*, ascorbate peroxidase (APX), catalase, dehydroascorbate reductase (DHAR), guaiacol peroxidase, glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), superoxide dismutases (SODs)) (De Vos et al. 1992;

Luna et al. 1994; Stohs and Bagchi 1995; Navari-Izzo et al. 1998; Gupta et al. 1999; Drazkiewicz et al. 2003; Wang et al. 2004; Lombardi and Sebastiani, 2005). The ascorbate-gluthatione cycle has been reported to be involved in response to excess Cu (Gupta et al. 1999; Drazkiewicz et al. 2003). The antioxidant responses have been observed in leaves and roots being either Cu concentration or time-dependent as well as plant specie or ecotype dependent.

The mechanism of Cu toxicity on photosynthetic electron transport has extensively also been studied *in vitro*, and it was found that PSII is the most sensitive site to Cu toxicity. Both the acceptor and the donor sides of PSII were suggested as the main targets of Cu toxic action. On the PSII reducing side, the  $Q_B$  binding site and the Pheo-Fe- $Q_A$  domain have been reported as the most sensitive sites for Cu toxicity (for review see Barón et al. 1995; Yruela 2005). The interaction of Cu toxicity with photoinhibitory and recovery processes on PSII has been also investigated (Yruela et al. 1996, Pätsikkä et al. 1998) demonstrating that Cu enhances the adverse effects of light. Considering that Cu is an efficient catalyst in the formation of reactive oxygen species (ROS), it was suggested that the increased Cu toxicity by light during photoinhibition is due to production of hydroxyl radicals (Yruela et al. 1996). A different proposal was given by Pätsikkä et al. (2002) suggesting that the reduced chlorophyll content observed in plant leaves grown in the presence of high Cu concentrations made leaves more susceptible to photoinhibition as a consequence of a Cu-induced Fe deficiency.

Susceptibility to excess Cu varies with plant species and ecotypes. For instance, alfalfa and barley are highly tolerant to excess Cu, but rice (*Oryza sativa*) and potato are less tolerant (Jones 1998). To better understanding such differences and how plants adapt to metal stress it is important to know how excess Cu affects gene expression.

DNA microarrays are powerful tools for providing an overview of gene expression under environmental conditions and in particular under Cu stress. Recently, several works have been done with this purpose. Weber et al. (2006) examined transcriptome changes upon  $Cd^{2+}$  and  $Cu^{2+}$  exposure



in roots of the Cd-hypertolerant metallophyte *A. halleri*. They did not find any evidence for Cu<sup>2+</sup>-specific responses. The overlap between *A. thaliana* and *A. halleri* was extensive. Most of the genes responsive to Cu<sup>2+</sup> in *A. halleri* were also found in the *A. thaliana* list. With very few exceptions, the genes of the '*Arabidopsis* Cu<sup>2+</sup> core response' were strongly responsive to many other abiotic stresses such as ozone, salt, cold and osmotic shock. This is likely due to the fact that excess Cu<sup>2+</sup> triggers the massive generation of reactive oxygen species, which is a consequence of most other biotic and abiotic stresses. Keinänen et al. (2007) identified genes that are up-regulated by Cu exposure in a Cu-tolerant birch (*Betula pendula* Roth.) clone. More recently, Sudo et al. (2008) examined gene expression in response to excess Cu in rice leaves. Microarray analysis revealed that Cu treatment particularly affects genes involved in defence, abiotic stresses, photosynthesis and transport. A large proportion of general and defence stress response genes are up-regulated under excess Cu conditions whereas photosynthesis and transport-related genes are down-regulated. The results suggest that the defence response has an essential role in the stress response to excess Cu. The defence-related genes involved in phytoalexin and lignin biosynthesis were the most sensitive to Cu. Defence-related genes could be effective targets for increasing Cu tolerance. Thus, the role of Cu as an antifungal agent may act in part by inducing defence-response genes, as well as by inhibiting the pathogen (Sudo et al. 2008). Additionally, plant management of abiotic and pathogen stresses had overlapping components, likely including signal transduction.

Proteomic approach has been also used to investigate the plant response to excess Cu. Bona et al. (2007) analyzed the root proteome of *Cannabis sativa* L., an annual herb with capability to absorb and accumulate heavy metals in roots and shoots, exposed to 150 µg g<sup>-1</sup> Cu<sup>2+</sup>. Cu up-regulated several proteins being the aldo/keto reductase the most up-regulated protein, which is a NAD(P)H-dependent enzyme widely distributed from mammals to insects, fungi and yeast. Its increase was associated to its involvement in detoxification process. Besides, actin, an important component of plant cytoskeleton and microfilaments, formate deshydrogenase (FHD), a

mitochondrial NAD-dependent enzyme which catalyzes the oxidation of formate into CO<sub>2</sub> maintaining a reduced environment, and the 40S ribosomal protein involved in protein synthesis machinery, were increased. In *Cannabis sativa* L., Cu also down-regulated proteins such as thioredoxin peroxidase, enolase, glutaredoxin and cyclophilin (Bona et al. 2007).

#### 2.4.3 Tolerance mechanisms to copper toxicity

In order to avoid metal toxicity all plants possess basal tolerance mechanisms, which appear to be involved primarily in avoiding the accumulation of toxic concentrations at sensitive sites within the cell preventing the damaging effects rather than developing proteins that can resist the heavy metal effects. The potential cellular mechanisms involved in tolerance include those by: *i*) reducing metal-uptake through mycorrhiza action or extracellular exudates; *ii*) immobilizing excess of Cu in the root and thus excluding the metal from the shoot; *iii*) stimulating the efflux pumping metal at the plasma membrane; *iv*) chelation of metals by phytochelatins, metallothioneins, organic acids or heat shock proteins; *v*) compartmentation of metals in the vacuole (Hall 2002; Krämer and Clemens 2006). There is little evidence that tolerant species or ecotypes show an enhanced oxidative defence; tolerant plants show rather enhanced avoidance and homeostatic mechanisms to prevent the stress (De Vos et al. 1991; Dietz et al. 1999).

Intraspecific and interspecific differences in sensitivity to Cu do occur between different plant species. On the other hand, with regard to mechanisms allowing Cu tolerance, a question of interest is whether this tolerance is constitutive in each species or depends on previous long-term exposure to metal. Van Thichelen et al. (2001) showed that some mycorrhizal species protect *Pinus sylvestris* L. against Cu toxicity extracellularly, although the amount of Cu retained by different fungi varies considerably. The mechanisms employed by the fungi are probably by binding to extracellular materials. Organic acids (citrate, malate, oxalate), carbohydrates, proteins or peptides enriched in cysteine or histidyl groups excreted by plants can facilitate metal uptake, but these

molecules can also inhibit metal acquisition by forming a complex with it outside the root that is not taken up. The importance of these mechanisms may vary in accordance with the concentration of metal supplied, plant specie or variety involved and the exposure time.

Dark deposits attached at the outer surface of the cell wall containing high level of Cu have been observed in plants grown under metal stress conditions (Vitória et al. 2006). Similar deposits were observed in Cu-stressed soybean cell suspensions that were accompanied by the accumulation of higher levels of citrate and malate (Bernal et al. 2006c). Similar levels of citrate and malate in copper tolerant *Nicotiana plumbaginifolia* L. cells were reported (Kishinami and Widholm 1987). Citrate appears to be responsible for Cu tolerance in *A. thaliana* (Murphy et al. 1999). Citrate synthesis was preferentially stimulated during the first time of Cu exposure in soybean cell suspensions, being one of the fastest responses to Cu exposure (Bernal et al. 2006c). Two organic acids exudation responses differing in time have been observed in roots of aluminium (Al) resistant plants (for review see Mariano et al. 2005). In the former response organic acids release is rapidly activated after Al exposure and the rate of release remains constant with time. In this case it has been suggested that Al activates a constitutive mechanism of organic acids transport in the plasma membrane and the activation of genes is not necessary. Al can activate anion channels, which have been proposed as the mediators of organic acids transport across the cell membranes. In the second one there is a delay in the organic acids release after the addition of Al and this release increases with time. In this case the activation of genes related to the metabolism and membrane transport of organic acids might be required.

Once inside the root cells, metals are translocated by membrane metal transporters and metal-binding proteins to their final destination. This process involves specific proteins (*i.e.*, metallothioneins, metallochaperones or low-molecular-weight metal chelators) that must maintain a fine balance between having enough essential metals available for metabolic functions and at the same time avoiding deficiency or toxicity. Excess metals are stored in a location where the metal can

do the least harm to cellular processes. This involves storage in special cellular compartments such as the vacuole. Sequestration may also be in the apoplast, or in specialized cells such as epidermal cells and trichomes.

Despite the widespread occurrence of metallothioneins (MTs) and the relatively high level of RNA expression of many MT genes, their function in plants remain poorly understood. Expression of some MT genes is induced by Cu: *i*) the level of expression of 2-type MT gene correlate closely with Cu tolerance in a group of *A. thaliana* ecotypes (Murphy and Taiz 1995); *ii*) expression of 2-type MT is elevated in a Cu-sensitive mutant that accumulates Cu (van Vliet et al. 1995); *iii*) Cu tolerance in the metallophyte plants *Silene vulgaris* (Moench) Garcke and *Silene paradoxa* L. was associated with increased levels of a 2b-type MT (van Hoof et al. 2001; Mengoni et al 2003); *iv*) the yeast MT *CUP1* gene introduced into tobacco plants contributed to Cu metal phytoextraction (Thomas et al. 2003). However, the involvement of MTs in Cu detoxification in plant has not been conclusively demonstrated. The divergence of plant MT protein sequences and the complex expression patterns of MT genes suggest that the functions of MTs may not be limited to Cu detoxification. Recently Guo et al. (2008) have reported direct evidence for functional contributions of MTs to metal homeostasis in plants.

The role of phytochelatin (PCs) in Cu detoxification has not been shown. Cu is a strong activator of PC biosynthesis but PC-deficient mutants show relatively little sensitivity to Cu. Since PCs can form complexes with Cu it could be possible that PC-Cu complexes are not sequestered in the vacuole (Cobbet and Goldsbrough 2002).

P<sub>1B</sub>-type Cu transporting ATPases are thought to be important not only in obtaining sufficient amounts of Cu ions for essential cell functions but also in preventing accumulations of these ions to toxic levels. On the other hand, Cu ions are chelated by specific chaperones and delivered to Cu pumps for transport into organelles or directly to cytosolic Cu dependent proteins. Thus, both Cu chelation and Cu pumping activity likely are not only required for Cu-uptake but also for

detoxification processes. Thus, these transporters and chaperones could be involved in the overall strategy of Cu tolerance. A possible role of metal transporters and chaperones in phytoremediation (defined as the use of green plants to remove pollutants from the environment or to render them harmless) has been proposed. Putative candidates to improve Cu phytoremediation include root Cu reductases and transporters, NA synthases and two Cu detoxification proteins: P-type ATPases and MTs (Puig et al. 2007). Concerning P-type ATPases, the Cu-tolerant plant *Silene vulgaris* (Moench) Garcke displays enhanced ATP-dependent Cu efflux across the root cell plasma membrane (Van Hoof et al. 2001). Furthermore, the inactivation of the ActP gene, which encodes a P-type ATPase, causes Cu hypersensitivity in *Rhizobium leguminosarum* and *Sinorhizobium meliloti* (Reeve et al. 2002). In *A. thaliana*, *AtHMA5* has been proposed as a candidate for overexpression to improve Cu detoxification (Andrés-Colás et al. 2006). More recently, Gao et al. (2009) have suggested the possible role of acyl-CoA-binding protein 2, ACBP2, and farnesylated protein *AtFP6* in mediating Cu, Cd and Pb transport in *A. thaliana* roots. *A. thaliana* plants overexpressing *ACBP2* or *AtFP6* were more tolerant to Cd than wild-type plants suggesting a similar role in Cu tolerance.

As mentioned above differences in sensitivity to Cu have been found among plant varieties. In order to understand the origin of such variations quantitative trait locus (QTL) analyses has been used to investigate the interaction between molecular mechanisms of Cu tolerance and phenotypic differences. A QTL study in *Arabidopsis* identified that Cu sensitivity was correlated with a major QTL on chromosome 1 (Kobayashi et al. 2008). This QTL1 regulates the Cu translocation capacity and involves the Cu<sup>+</sup>-transporting P<sub>1B</sub>-type ATPase *AtHMA5*. The results revealed that amino acid polymorphisms in strictly conserved motifs of *AtHMA5* are involved in Cu tolerance of the roots and explain Cu tolerance variations in *Arabidopsis*. The same mechanism (*i.e.* substitution of amino acids in conserved domains) was identified as a cause of dysfunctional Cu homeostasis in human Menkes and Wilson diseases. This may support the importance of HMA5 in Cu tolerance and therefore the importance of Cu homeostasis in Cu tolerance. Similar mechanisms may contribute to

natural variation in plant tolerance to Cu, especially for root growth, but further studies are necessary to clarify that.

### 3 INTERACTIONS BETWEEN COPPER AND OTHER ELEMENTS

In general transition metal ions are distinguished by their different chemical properties, *i.e.* redox potential, coordination geometry, charge and thermodynamic and kinetic properties of ligand exchange. Accordingly, in a given metalloenzyme, a specific metal ion is used for a specific function. However, according to the Irving-Williams series ( $Zn^{2+} < Cu^{+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$ ) metal ions can bind to organic ligands in a metal-binding site of a metalloprotein, metal-chaperone or metal transporter with different affinities (Fraústo da Silva and Williams 2001). Thus, although binding affinity for a metal ion is also determined by other secondary factors such as the size of metal binding-site cavity in a protein, the geometry of ligand atoms and other characteristic, normally each metal ion can be replaced by other metal ion downstream in the Irving-Williams series. A further implication of the chemical principles illustrated by the Irving-Williams series is that metal homeostasis of one transition metal should generally not be considered alone, but always in the context of all cations and their respective concentrations. Cations chemically similar to other can enter into plants by competing with uptake pathways for macronutrients and micronutrients metal ions. Consequently in metal-polluted areas toxic metal ions can enter into most plants since metal homeostasis network are not equipped to avoid the entry of non-essential metal transitions at high concentration. Therefore, one major mechanism of toxic action of all transition metal is the efficient competition of metal ions for specific binding sites, consequently, displacements of essential metal ions from their binding sites can occur. For instance, it has been shown that the central ion  $Mg^{2+}$  in chlorophyll was substituted by Cu and other toxic metals under metal excess conditions resulting in an impairment of the correct function of the

chlorophyll-complexes (*i.e.* light harvesting antenna complex) because metal-substituted chlorophylls are not suitable for photosynthesis (Küpper and Kroneck 2005).

In plants relatively little is known about Cu transport into and within cells showing a dependence on Cu for Fe, Zn, Mn and other element assimilation. Schmidt (1999) reported that Cu and Fe compete in ion-uptake. Pätsikkä et al. (2002) observed that excess Cu in hydroponic medium induces a Fe-deficiency in bean plants. Chen et al. (2004) observed that Fe-deficiency induces Cu accumulation in *Commelina communis* L. plants. Furthermore, Rombolà et al. (2005) found that Fe-deficiency increases the Cu content and decreases the Zn content in leaf blades of sugar beet grown hydroponically. Cu and Fe antagonism often occurs in plants grown under Cu toxicity (Foy et al. 1978; Wallace and Cha, 1989; Lombardi and Sebastiani 2005). Nevertheless, opposite scenario has been also observed in oregano (Panou-Filotheou et al. 2001), rice seedlings (Kitagishi and Yamane 1981) and wheat (*Triticum aestivum* L. cv Vergina) (Lanaras et al. 1993) plants exposed to Cu toxicity in soil. An increasing concentration of soil Cu resulted in a parallel increase in leaf Cu content with no reduction in the leaf Fe and Mg. These apparently contradictory results may be explained by different tolerance strategies adopted by different plants.

Other organisms such as mammal cells, yeast or certain algae do not appear to manifest a competition showing a dependence on Cu for Fe assimilation (Franklin et al. 2002). A Cu dependent Fe assimilation pathway has been found in the unicellular green alga *Chlamydomonas reinhardtii* (La Fontaine et al. 2002). Additionally, an antagonist interaction between Cu and Zn was observed in this alga (Herbik et al. 2002). Similar feature has been observed in some plants. Soil Cu affected negatively the accumulation of Zn in roots of oregano (Panou-Filotheou and Bosabalidis 2004). More recently, Bernal et al. (2007a) demonstrated that Cu interacts differently with Fe and Zn depending on the pathway through excess Cu is supplied. Thus, soybean plants treated with excess Cu through leaves behave differently than plants treated by supplementing the growth medium with excess Cu. Soybean plants showed no antagonist interaction between Cu- and Fe-uptake when

excess Cu was supplied through leaves but Cu compete with Fe-uptake in plants grown with excess Cu in the hydroponic medium. Concerning Zn-uptake soybean plants exhibited Zn content decrease upon Cu treatment of leaves whereas the opposite was observed upon Cu treatment through roots. Interestingly, plants with Cu-treated leaves behaved similarly as soybean cell suspensions grown in the presence of excess Cu (Bernal et al. 2006b, 2006c). The different plant response observed upon these two Cu-treatments might be explained assuming different Cu-uptake strategies in leaf and root cells.

#### 4 PROSPECTS

This review shows that progress in understanding Cu homeostasis in plants has been noticeable in last decade, but there are still unclear aspects or little investigated. For instance, several families of genes involved in regulation of Cu homeostasis have been identified and their expression analyzed under either deficiency or excess Cu conditions, but little is known about the structure and functional mechanisms of proteins generated by those genes. Some of these proteins are inserted in the membranes of cells and organelles, and it is true that structural studies on membrane proteins are difficult but further biochemical and structural studies including molecular interactions and molecular recognition of proteins involved in Cu homeostasis should be necessary to know the molecular basis of Cu trafficking and transport.

On the other hand, most of molecular studies are focused in intracellular homeostasis being the information on Cu distribution and remobilization in xylem and phloem saps as well as Cu xylem-to-phloem exchange less available. Furthermore, researchers should pay more attention to metal interactions by their relevant implications in Cu distribution and remobilization within the plant.

Other interesting aspect to further explore is the putative significant role of Cu chaperones and transporters in Cu tolerance. Recently, it has been point out the role of *AtHMA5* transporter,



involved in Cu transport in roots, as important element in Cu tolerance mechanisms but it is unknown if other proteins involved in Cu transport and trafficking are also candidates. Moreover, variations in Cu tolerance exist among plant varieties however the involvement of specific genes related with Cu homeostasis in determining such variations have been little investigated. Studies of natural variations based on quantitative trait locus (QTL) analysis have provided a useful approach to understand the mechanisms of variation in target traits such as freezing tolerance, salinity tolerance, growth and flowering among others. Recently, this approach has been applied in *Arabidopsis* to identify critical genes regulating variations in Cu tolerance (Kobayashi et al. 2008). The results revealed that amino acid polymorphisms in certain genes involved in Cu transport can be responsible of such variations. These studies suggest that the combination of association mapping analysis and the biochemical approach could be useful to identify key genes regulating variations related to Cu tolerance or sensitivity among genotypes and varieties. An interesting question for future research is if the same mechanisms found in *Arabidopsis* can explain variations in Cu tolerance of other crop species. The understanding of such mechanisms may be used in molecular breeding programmes (*i.e.* marker-assisted selection).

## 5 CONCLUSION

In summary, our knowledge of Cu and heavy metals homeostasis is still rudimentary in some cases. A comprehensive understanding of Cu transport and trafficking across plant membranes and distribution and remobilization through xylem and phloem saps, at the molecular level, including metal interactions as well as variations in Cu tolerance and sensitivity in plants will be essential for developing schemes to genetically engineer plants that accumulate specific metals, either for use in phytoremediation or to improve human nutrition.

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## FIGURE LEGENDS

Figure 1. Scheme of transport pathways identified for Cu in a generic plant cell. Cu-membrane transporter proteins are indicated in orange, Cu-chaperones in violet, and Cu-proteins in blue.

Arrows indicate the proposed direction for metal transport. CCH, copper chaperone; ATX1, antioxidant 1; CCS, copper chaperone for Cu/Zn superoxide dismutase; CSD1, cytosolic Cu/Zn superoxide dismutase; CSD2, chloroplastic Cu/Zn superoxide dismutase; CSD3, peroxisomal Cu/Zn superoxide dismutase; COPT, copper transporter; COX, cytochrome-c oxidase; ER, endoplasmic reticulum; FRO, ferric reductase oxidase; HMA, heavy metal P-type ATPase; MT, metallothioneins; NA, nicotianamine; PAA, P-type ATPase of *Arabidopsis*; Pc, plastocyanin; RAN1, responsive-to-antagonist 1; SOD, superoxide dismutase; YSL, yellow stripe-like protein; ZIP, IRT-like protein. Scheme modified from Pilon et al. (2006), Puig et al. (2007), Bernal (2006a).

Figure 2. Predicted membrane topology for several members of COPT and P<sub>1B</sub>-ATPase (HMA) families Cu-transporters. This topology is based on predictions but has not been verified experimentally.



TABLE 1. Copper homeostasis proteins in plants

Family	Name	Description	Subcellular localization	Tissue expression	Referentes
ZIP	<i>AZIP2</i>	Divalent cation transporter	Plasma membrane?	Root	Grotz <i>et al.</i> (1998); Wintz <i>et al.</i> (2003)
	<i>AZIP4</i>	Divalent cation transporter	Plasma membrane?		
	<i>MZIP4</i>	Divalent cation transporter		Root, leaf	López-Millán <i>et al.</i> (2004)
COPT	<i>AICOPT1</i>	High-affinity Cu <sup>+</sup> transporter	Plasma membrane?	Root, pollen, embryo, stomata, trichome	Kampfenkel <i>et al.</i> (1995); Sancenón <i>et al.</i> (2004)
	<i>AICOPT2</i>	High-affinity Cu <sup>+</sup> transporter	Plasma membrane?		Sancenón <i>et al.</i> (2003)
	<i>AICOPT3</i>	High-affinity Cu <sup>+</sup> transporter	Chloroplast?		
	<i>AICOPT5</i>	High-affinity Cu <sup>+</sup> transporter	Secretory pathway?		
	<i>AICOPT6</i>	High-affinity Cu <sup>+</sup> transporter			
P <sub>1B</sub> -ATPase	<i>AHMA1</i>	Cu <sup>2+</sup> -P <sub>1B</sub> -ATPase transporter?	Chloroplast envelope	Root, shoot	Seigneurin-Berny <i>et al.</i> (2006)
	<i>AHMA5</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Secretory pathway?	Root, flower, pollen	Andrés-Colás <i>et al.</i> (2006)
	<i>OshMA5</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Secretory pathway?	Root	Sichul <i>et al.</i> (2007)
	<i>AHMA6(PAA1)</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Chloroplast envelope	Root, shoot	Shikanai <i>et al.</i> (2003); Abdel-Ghany <i>et al.</i> (2005b)
	<i>OshMA6</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Chloroplast envelope	Root, shoot, leaf	Sichul <i>et al.</i> (2007)
	<i>AHMA7(RAN1)</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Trans-Golgi network?		Hirayama <i>et al.</i> (1999); Woeste and Kieber (2000; Chen <i>et al.</i> (2002)
	<i>BrHMA7(BrRAN1)</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Trans-Golgi network?		Southron <i>et al.</i> (2004)
	<i>OshMA7</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Trans-Golgi network?	Root, shoot, leaf	Sichul <i>et al.</i> (2007)
	<i>AHMA8(PAA2)</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Thylakoid membrane	Shoot	Abdel-Ghany <i>et al.</i> (2005b)
	<i>GmHMA8</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Thylakoid membrane	Leaf, mesophyll cell	Bernal <i>et al.</i> (2007b)
	<i>OshMA8</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Thylakoid membrane	Root, shoot, leaf	Sichul <i>et al.</i> (2007)
<i>OshMA9</i>	Cu <sup>+</sup> -P <sub>1B</sub> -ATPase transporter	Plasma membrane	Vascular tissue (phloem, xylem), mesophyll tissues, anthers	Sichul <i>et al.</i> (2007)	
ATX	<i>AICCH</i>	ATX1-like Cu chaperone	Cytosol	Stem, vascular tissue	Himelblau <i>et al.</i> (1998); Andrés-Colás <i>et al.</i> (2006)
	<i>AiATX1</i>	ATX1-like Cu chaperone	Cytosol		Puig <i>et al.</i> (2007)
	<i>LeCCH</i>	ATX1-like Cu chaperone	Cytosol		Company and González-Bosch (2003)
CCS	<i>AiCCS</i>	Chaperone for Cu/ZnSOD	Cytosol and chloroplast	Stem, flower, leaf	Abdel-Ghany <i>et al.</i> (2005b); Chu <i>et al.</i> (2005)
	<i>LeCCS</i>	Chaperone for Cu/ZnSOD			Zhu <i>et al.</i> (2000)
	<i>SiCCS</i>	Chaperone for Cu/ZnSOD			Trindade <i>et al.</i> (2003)
	<i>ZmCCS</i>	Chaperone for Cu/ZnSOD			Ruzsa and Scandalios (2003)

	<i>GmCCS</i>	Chaperone for Cu/ZnSOD	Chloroplast	Mesophyll cells, leaf	Sagasti S, Bernal, M, Picorel R, Yruela I, unpublished results
COX	<i>AtCOX17-1</i> <i>AtCOX17-2</i>	COX17-like Cu chaperone COX17-like Cu chaperone			Baladin and Castresana (2002); Wintz and Vulpe (2002) Baladin and Castresana (2002); Wintz and Vulpe (2002)
YSL	<i>ZmYS1</i> <i>AtYSL1</i> <i>AtYSL2</i> <i>OsYSL2</i> <i>AtYSL3</i> <i>TcYSL3</i>	Cu <sup>2+</sup> -NA complex transporter? Cu <sup>2+</sup> -NA complex transporter Cu <sup>2+</sup> -NA complex transporter? Cu <sup>2+</sup> -NA complex transporter? Cu <sup>2+</sup> -NA complex transporter Cu <sup>2+</sup> -NA complex transporter?	Plasma membrane? Plasma membrane Plasma membrane? Plasma membrane Plasma membrane? Plasma membrane	Root, shoot Pollen, vascular tissue, peduncle, leaf Root (endoderm phericycle), shoot Leaf (phloem), seed Pollen, flowers, root, leaf Root	Roberts <i>et al.</i> (2004); Schaaf <i>et al.</i> (2004) Waters <i>et al.</i> (2006); Curie <i>et al.</i> (2009) DiDonato <i>et al.</i> (2004); Schaaf <i>et al.</i> (2005) Koike <i>et al.</i> (2004) Waters <i>et al.</i> (2006); Curie <i>et al.</i> (2009) Gendre <i>et al.</i> (2007); Curie <i>et al.</i> (2009)

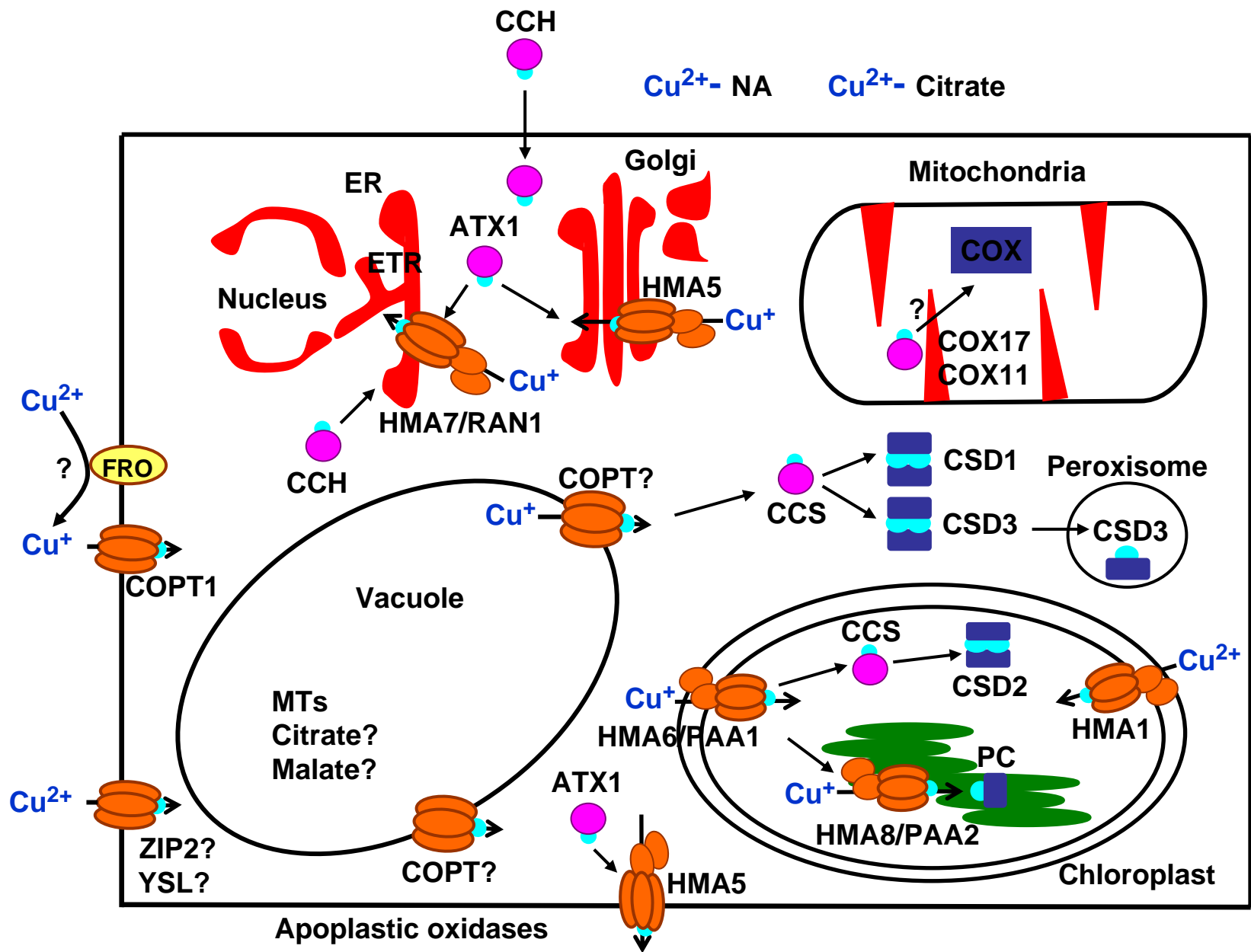
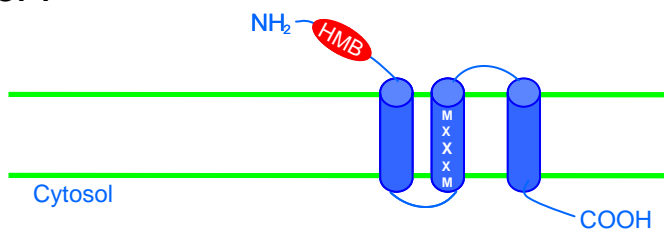
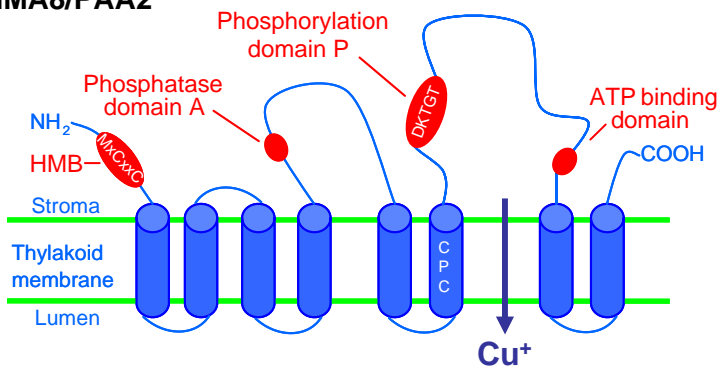


Figure 1

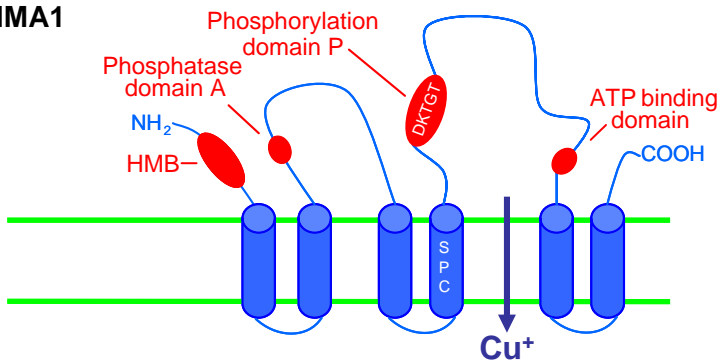
### COPT



### HMA6/PAA1 HMA8/PAA2



### HMA1



### HMA7/RNA1 HMA5

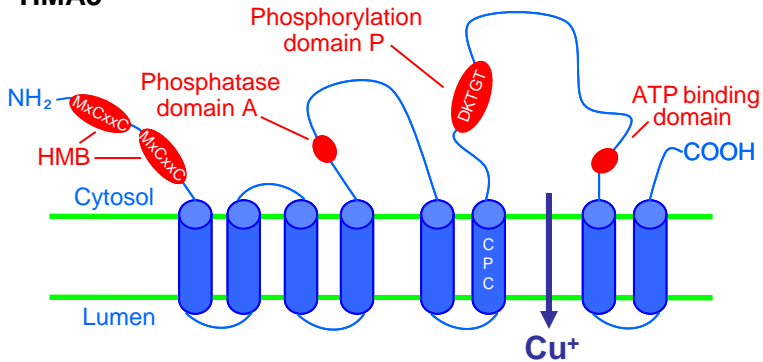


Figure 2