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Review

Throwing Copper Around: How Plants Control Uptake, Distribution, and Accumulation of Copper

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Abstract: Copper (Cu) is essential to plants but can also be harmful due to Fenton chemistry. Because of that, it is necessary to keep Cu within a narrow concentration limit. Plants evolved mechanisms to sense Cu and precisely regulate uptake and accumulation to avoid both deficiency and toxicity. However, plants experience variable Cu levels in soils, both due to natural environments as well as human's-based farming practices that can lead to Cu accumulation in soils. Therefore, we need to understand Cu homeostasis. Here, we review how Cu is found in soils; Cu toxicity responses in plants; the role of Cu transporters, described mainly in model species such as *Arabidopsis thaliana* and *Oryza sativa*; the crosstalk between Cu and iron (Fe) homeostasis; Cu hyperaccumulator plants; and discuss some gaps and future directions, which can improve our understanding in the field.

Keywords: copper; transporter; nutrient uptake; metal toxicity; nutrient crosstalk

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1. Introduction

The transition metal copper (Cu) is essential for most living organisms, including higher plants [1,2]. Under physiological conditions, Cu is found in two forms, the reduced Cu^{+} state (cuprous) and the oxidized Cu^{2+} state (cupric) [3]. Depending on the state, Cu can bind different substrates [4,5], from sulfur-containing compounds having a thiol or a thioether group to oxygen or imidazole nitrogen groups [6,7]. Such dual chemistry of Cu allows the interaction with several molecules [8], especially enzymes that catalyze redox reactions, driving a myriad of biochemical pathways [2,9,10]. On the other hand, the redox capacity of Cu can directly lead to the generation of ROS (reactive oxygen species) through Fenton and Haber–Weiss reactions, causing severe damages to proteins, DNA, and other biomolecules [2,4,10–12]. Besides being essential in small amounts to ensure cellular functions, Cu excess may exert detrimental effects on plant primary production and even survival [10,13,14]. Therefore, it is essential for a plant to have a strictly controlled Cu homeostasis [2,4,15].

Plants use Cu as a cofactor for a wide range of proteins involved with several physiological processes, including photosynthesis, mitochondrial respiration, carbohydrate

metabolism, formation of phenolics in response to pathogen attack, superoxide scavenging, cell wall remodeling, and ethylene perception [2,5,10,14–16]. The majority of Cu proteins (~90%) is found in nature function as oxidoreductases [17,18]. The most abundant Cu protein in plants is plastocyanin (which utilizes about 50% of the Cu in the plastids), a protein essential for photosynthetic electron transport in chloroplasts that transfers electrons from the cytochrome b6f complex to photosystem I (PSI) [1,4,5,13,16,19]. Cu is part of Complex IV of the mitochondrial respiratory chain, and thus, is involved in metabolic pathways that supply energy for cellular processes [13,20]. Other Cu proteins are Cu/Zn superoxide dismutase (dismutation of superoxide), laccase (cell wall remodeling), amine oxidase (cell wall and plant tissue differentiation, wound healing, and response to pathogens), and polyphenol oxidase (wounding and pathogen response) [1,2,4,5,15,21,22]; for a comprehensive review, see Andresen et al. [18].

As a trace element, an optimal quantity of Cu is required to ensure proper cellular function, but in excess, it induces harmful impact on the primary production and survival of plants [4]. For this reason, both Cu deficiency and Cu excess can bring severe consequences to plants. Therefore, it is essential to ensure adequate Cu uptake and distribution in order to minimize its deleterious phytotoxic effects that in turn would regulate various homeostatic processes at cellular and whole plant levels [23,24]. Here we provide a comprehensive review about the Cu homeostasis, including its chemistry in the soil, plant responses and tolerance mechanisms to Cu toxicity, the known genes involved in Cu transport and regulation in model plants, and the rare hyperaccumulation cases. We also discuss gaps in the field and future directions.

2. Copper in Soils

Copper is a metal element and its content in agricultural soils is dependent on the concentration of parent materials, climate conditions, pedological factors, and addition through human activities, such as applications of foliar fungicides, as well as mineral and organic fertilizers [24–27]. Pollution by Cu in agricultural soils is reported in several parts of the world, especially in vineyards and orchards soils [24,27–29]. Copper in soils is found at different oxidation states, predominantly in divalent form (Cu^{2+}) in acidic soils, but also in monovalent form (Cu^{+}) and even in its fully reduced form (Cu^{0}) [30]. Copper added via external sources interacts with the mineral and organic components of the solid phase through chemical bonding at different energy levels. Copper is initially retained in ion exchange sites (non-specific adsorption), where ions are hydrated and adsorbed by electrostatic forces, resulting in low stability to the interaction. After this initial stage, slower reactions occur, whose balance will depend on the amount and the time of Cu application, and may progress to ionic and covalent bonds (specific adsorption) with higher energy level and stability [31,32]. In the specific adsorption in functional groups of inorganic particles, Cu partially or completely loses its hydration water, forming the inner sphere complex with the surface of Fe, Mn, Al oxides, uncrystallized aluminosilicates, and edges of clay minerals that present OH^{-} or an H_2O molecule bound to a metal ion of the crystal lattice [33–35].

The electron configuration of Cu $[Ar]3d^{10}4s^{1}$ provides high adsorptive reactivity to organic compounds, promoted by the presence of functional groups containing S and N, carboxylic groups (COOH), and (to a lesser extent) phenolic compounds [30,36]. As a consequence of this high interaction between Cu and the components of the soil solid phase, there is accumulation in the surface layer of soils that are not mobilized for long periods, increasing the risk of toxicity to plants, as most of the root system is concentrated in this layer [27,29,35]. In addition to Cu binding with the solid phase, Cu lability in soil is changed by physical and chemical characteristics such as levels of minerals, oxides, and hydroxides of Fe, Al, and Mn, as well as carbonates and organic matter, soil pH, and cation exchange capacity (CEC) [30,33].

The type and energy level of the adsorptive binding of Cu with the solid phase will determine the reversibility degree of the reactions, directly influencing Cu availability. The

main reactions that control Cu availability in soils are precipitation/dissolution, adsorption/desorption, and oxidation [28,34]. These reactions will determine Cu concentration in the soil solution. Although the concentration in the soil solution represents a small fraction of the total soil content, it is in equilibrium with the fractions sorbed in the solid phase and is of great importance in Cu uptake and bioavailability [32,37,38]. The successive additions of Cu promote the saturation of the most avid adsorption sites and increase Cu concentration in the soil solution [39]. This increases the risk of toxicity to plants, but also warns to the potential of transfer and contamination of water sources [40,41]. In the soil solution, Cu can be found in ionic form or bound to organic compounds or anions. The formation of complexes and ionic pairs in the soil solution favors desorption and increases Cu mobility in the soil profile, promoting accumulation in lower layers [39].

Plants and microorganisms absorb Cu of the soil solution preferably in inorganic chemical form, which is coordinated by water molecules, and classified as free species (Cu^{2+}) [30]. Thus, increasing the proportion of chemical species of Cu complexed in the soil solution reduces its bioavailability. The distribution of the chemical species of Cu in the soil solution is governed by the characteristics of the soil and solution, especially by Cu content, dissolved organic compound concentration, and pH [29,42]. The chemical species of Cu predominant in the solution of cultivated soils are free species and those complexed with organic compounds. However, other species complexed with inorganic binders (OH^-, CO_3^{2-}, PO_4^-, SO_4^{2-}, and NO_3^-) are typically found in low proportions [39,42,43]. The high affinity of soluble Cu to dissolved organic ligands and the formation of more stable complexes indicate that production systems with high amounts of organic residues or even soil regions such as the rhizosphere (where organic acid concentration is normally high), Cu species complexed in the soil solution tend to predominate, with low bioavailability and potential toxicity to plants [38].

Plants grown in Cu-contaminated soils may express adaptive mechanisms when exposed to stress conditions, changing Cu bioavailability in the rhizosphere [38]. The scale of the effects of the plants, associated with soil microbiota on Cu bioavailability, is higher in the rhizosphere. The reduction in Cu bioavailability may occur because of increased exudation of low molecular weight organic acids and pH, which increase the proportion of complexed chemical species and, consequently, reduce Cu^{2+} [38,44,45].

3. Plant Responses to Copper Toxicity

Since plants are sessile, they cannot escape locally high metal concentrations [18]. Critical reviews on mechanisms of metal toxicity in plants were recently published [18,46–49]. High Cu concentration is toxic to plants, causing significant negative effects ranging from morphological and physiological to molecular level, and are evident at all stages of plant growth [50]. Under extremely high Cu concentrations, both high- and low-affinity binding sites for Cu will be occupied, making the effect of Cu toxicity non-specific [18]. Unspecific metal tolerance mechanisms include: (1) suberin accumulation and lignification of the root cells, preventing metal translocation to the shoot [51]; (2) enhanced ROS defense and metal detoxification via phytochelatins in roots and shoots [48]; (3) sequestration of toxic metal ions by transport and storage in cell compartments where they interfere less with metabolic processes, such as the vacuole [18,48]; (4) binding of divalent/trivalent ions to negatively charged polysaccharides such as pectin and hemicellulose of the cell wall [52,53]; and (5) increased deposition of callose and remodeling of the cell wall, enhancing the binding capacity and sequestration within the apoplast [54,55]. The majority of plants use one or a combination of these mechanisms to cope with heavy metal excess [48].

3.1. Effects of Copper Excess on Seed Germination

Germination testing is effective to determine Cu toxicity effects on different plant species [50]. Excess Cu in soil significantly reduces seed germination in many plant species, probably due to a restriction in the breakdown of reserve food resources (i.e., starch and sucrose) by inhibiting activities of α-amylase and invertase isoenzymes [56]. Germination

experiments were conducted on wheat (*Triticum aestivum* L.), pea (*Pisum sativum*), and tomato (*Solanum lycopersicum* L.) under Pb, Cd, and Cu exposure. Irrespective of the tested crop seeds, Cu revealed the highest deleterious effect (51.2%) on germination, followed by Pb (47.5%) and Cd (35.3%). Higher toxicity to Cu in germination might be due to greater Cu permeability of the embryo cover or due to higher Cu impact on the activity of amylase and protease enzymes, causing inhibition of food supply to the growing radicle and plumule [57]. In addition, altered overall metabolism and water transport are linked with Cu toxicity in the seed germination process [58].

3.2. Effects of Copper Excess on Anatomical/Morphological Changes

Negative effects of Cu excess appear in the form of morphological, anatomical, and biochemical responses, among which the anatomical changes show the most relevant effects [59] (for a comprehensive review, see Yadav et al. [49]). As a general response, inhibition of root growth and architecture has been observed in several plant species when grown in the presence of high metal concentrations. Enhanced lateral root formation was observed in Cu-exposed Arabidopsis and radish (*Raphanus sativus*) plants. However, decreased mitotic activity, earlier senescence and low cell viability were typical for affected meristems of newly formed lateral roots because they remained short and probably non-vital [60]. Similarly, the production of short low-viable lateral roots in radish was observed together with the subero-lignification of root tip, which probably limited primary root growth as well as lateral roots [61]. Additionally, Cu severely decreased total root volume of oregano (*Origanum vulgare*) plants, besides provoking an extensive malformation in root parts, caused by disorganized/folded cell walls of rhizodermis, whose proportion was decreased by 55% compared to control [62]. Additionally, a decrease in cortical and an increase in endodermal cell wall thickness were detected in Cu-treated maize [63] and bean [64] roots, respectively. The central cylinder and root vasculature can also be affected by Cu stress. In oregano plants, xylem vessel diameter was increased without changing the cell wall thickness, while in phloem cells the opposite was found [62]. In beans, both xylem and phloem cell walls thickness increased after Cu stress, probably caused by a massive lignin deposition [64].

Anatomical alterations caused by high Cu concentrations have also been observed in sorghum (*Sorghum bicolor*) stems, with a reduction in the number of vascular bundles [65], as well as in oregano leaves, with an increased thickness, stomatal frequency, number of hairs, and volume of mesophyll cells [66]. On the other hand, reduced stomatal frequency after Cu exposure was observed by Kasim (2006) [65] in sorghum leaves, along with decreased leaf vascular tissues. Copper also reduced the size of conductive tissues of maize [63], and induced the thickening of xylem tissues/reduction of perivascular fibers in beans [64].

It is obvious that such anatomical alterations are followed by morphological changes. The effects of high Cu concentrations in plant morphology have been studied in different plant species, and the most common alterations include decreased root/shoot/leaf lengths, leaf area, stomatal conductance, and stem size, along with browning/rotting of the roots, reduced number of roots per plant, and leaf chlorosis [5]. Considering all these Cu-induced changes, it is not surprising that numerous studies have demonstrated the toxic effect of high Cu concentrations on the biomass and grain yield of food crops grown both in hydroponics or soil conditions (for a comprehensive review, see Adrees et al. 2015 [50]).

3.3. Effects of Copper Excess on Photosynthetic Apparatus

When compared with other potentially toxic essential trace elements, such as excess Mn, Zn, and Cd, excess Cu is more toxic to plants [50,67–69]. Under nanomolar to micromolar range, Cu toxicity can damage the photosynthetic apparatus. Copper toxic effects on chlorophyll biosynthesis have been reported in a number of crop plants [50,70–74]. Leaf chlorosis is a primary symptom of Cu toxicity [75]. Reduction in photosynthesis is related to decrease in chlorophyll contents and structural damages to the photosynthetic apparatus [23], with PSII being more sensitive than PSI [47,56]. The deleterious effects of excess

Cu on chlorophyll content may be linked with decline in P and Fe levels in shoots [76]. Under high light, Cu inhibits the PSII reaction center, and under low light, it inhibits the light-harvesting complex II (LHCII) antenna systems [77–79], both by replacement of the Mg^{2+} ions in the chlorophylls by Cu^{2+}, which cannot drive photosynthesis [80].

Copper excess can also impair chloroplast structure and thylakoid membrane composition [81–83]. Disturbed metabolic activities, such as loss of chloroplast integrity, change in plastid membrane composition, and inhibition of photosynthetic electron transport, have also been evidenced in plants exposed to elevated Cu levels [2,50,84]. The hampering of the photosynthetic electron transport enhances the 'leakage' of electrons and generates ROS on both the donor and acceptor side of PSII [85]. Copper excess can also cause a reduction in photosynthesis mainly as a consequence of higher photoinhibition [50,86].

3.4. Effects of Copper Excess on the Uptake and Accumulation of Other Mineral Nutrients

A common effect of Cu toxicity in plants is decreased uptake and accumulation of other mineral nutrients [50,87]. Under high Cu in either nutrient solution or in soil, Cu concentration is markedly higher in roots than in shoots and leaves, and such high Cu accumulation in roots can negatively affect the uptake and accumulation of other nutrients (Ca, Mg, K, N, P, Zn, Mn, Co, and Fe) in several plant species. On the other hand, Cu accumulation can also positively affect the uptake and accumulation of Ca, Mg, K, Mn, S, and Fe (for a comprehensive review, see Adrees et al. 2015 [50]). Therefore, the Cu effect on plant mineral uptake and accumulation clearly depends on the plant species, Cu concentration in the root medium, exposure duration, and growth conditions.

While most of these negative or positive effects are not molecularly/physiologically investigated in depth, it is important to highlight that Cu is highly toxic to Fe concentration as compared to other nutrients, which suggests an antagonistic relationship between Fe and Cu [50,88] (please see sections of Fe-Cu crosstalk below). Since Fe is needed for cytochrome complexes, ferredoxin, and intermediates of the thylakoid electron transport chain [12], it is evident that Cu-induced limitation of Fe influences photosynthetic performance [18]. The complex dynamics of nutrient uptake and accumulation under Cu stress might be due to competition between Cu and nutrients for transporters, alterations in the expression of genes participating in nutrient uptake at transcriptional or post-transcriptional level, and variations in plasma membrane permeability [2,89–91].

3.5. Plant Tolerance to Copper Toxicity

The classical Cu tolerance mechanisms include reduced Cu uptake by the roots, limited Cu translocation from the roots to the shoots, efficient metal efflux through the plasma membrane, chelation of Cu with organic molecules, stimulation of phytochelatins, metallothioneins and heat shock proteins, and Cu binding to cell walls [10,50,56,70,71,73,92]. These strategies may limit Cu toxicity by either reacting directly with or restricting Cu uptake in plants [93].

The first defense mechanism of reducing toxicity from excess Cu is by decreasing or preventing uptake from the soil, either by chelating or precipitating Cu ions in the rhizosphere [56]. Different studies reported that organic acids (such as citrate and malate) secreted by the roots reduce metal absorption via chelation and complexation [94,95]. Following metal uptake, excess Cu accumulation must be either limited to roots (to avoid the accumulation of toxic Cu concentrations at sensitive tissues, such as the leaves) or compartmentalized into less sensitive vacuoles and organs [48,56]. Once inside the plant (in both roots and shoots), excess Cu induces the secretion of certain organic acids, such as proline, histidine, and citrate, and also other compounds which can chelate high Cu levels, such as phytochelatins, metallothioneins, proteins, cellulose, hemicellulose, and polysaccharides [4,10,44,50,93,96,97]. The accumulation of secondary metabolites (such as betacyanin) can also contribute to Cu tolerance, as demonstrated by Morales et al. (2012) [98]. Plants can also tolerate excess Cu as sulfur-coordinated Cu^{+} species resembling glutathione/cysteine-rich proteins, both in roots and leaves [99], changing the cell wall

composition [100], or still modulating the synthesis of nitric oxide [23], which maintains the cellular redox homeostasis interfering with the activities of antioxidant enzymes. Yet, excess Cu can be stored in subcellular compartments, which are less sensitive to toxic effects, such as the cytosol, chloroplast, and vacuole [4].

4. Copper Homeostasis in Model Plant Species

Copper homeostasis must be tightly controlled by plants, given its essentiality and redox properties that can make Cu prone to produce ROS. Therefore, both Cu deficiency and excess can be harmful. Here, we review the current knowledge of Cu homeostasis, including transporter and transcription factors involved in regulating its uptake, distribution, and accumulation, with special focus on the model plant species *Arabidopsis thaliana* and rice.

4.1. Regulation of Copper Uptake in Roots

Copper can be found in soils either as Cu^{+} and Cu^{2+} [2]. Most understanding of Cu soil uptake is derived from describing how plants respond to Cu deficiency in *A. thaliana*. The transcription factor AtSPL7 (SQUAMOSA promoter-binding protein-like, or SPB-like) is the main regulator of root responses to low Cu [101]. *AtSPL7* is not transcriptionally regulated by varying levels of Cu. It is suggested that when Cu is present at sufficient concentration, it binds AtSPL7 and decreases its DNA affinity to SBP-like binding motifs, whereas low Cu levels inside the cell increases such affinity, up-regulating Cu deficiency transcripts [102]. This model is supported by how *Chlamydomonas reinhardtii* orthologous protein Cu response regulator 1 (CRR1) regulates its downstream genes (for a comprehensive review on algae Cu homeostasis, please see Merchant et al. (2020) [103]). Evidence for post-translational regulation of AtSPL7 suggest that it can interact with AtKIN7, a nuclear-localized protein that might be involved in Cu deficiency response [104]. AtSPL7 was also shown to interact with AtHY5, a central regulator of photomorphogenesis in *A. thaliana*, as their target genes extensively overlap, with miRNA408 integrating light and Cu responses [105]. Moreover, it was also shown that AtSPL7 is involved in integrating Cu homeostasis with the circadian clock, showing that Cu can affect the daily oscillation of clock genes [106].

AtSPL7 directly regulates several genes important for the so-called "Cu economy response", in which plants become more efficient in saving Cu for essential proteins, such as plastocyanin, while switching off non-essential ones (Figure 1). For that, AtSPL7 binds to Cu responsive elements (CuRE), which are found in several Cu deficiency-regulated genes [4,101,107]. Among the well-established targets are the primary root uptake transporters of the COPT gene family (see below), which likely perform primary Cu uptake from rhizosphere; ferric superoxide dismutase (FeSOD), which can perform similar role as Cu/ZnSODs but using Fe as a cofactor, therefore saving Cu for essential functions; and a set of microRNAs (397, 398, 408, and 857) which down-regulate the expression of cupric proteins, such as Cu/ZnSOD, Cu chaperones (such as CCS, which delivers Cu to Cu/ZnSODs), laccases, plantacyanin, and others [108–111]. MicroRNAs 397, 398, and 408 are well conserved in plants, as well as their potential targets, suggesting conservation of this regulatory network, which can work as a diffusible signal between symplastically connected cells to adjust Cu usage [111]. Interestingly, the Cu economy response seems to be at least partially conserved in rice plants [112], besides the fact that no ortholog to AtSPL7 is described in rice to date.

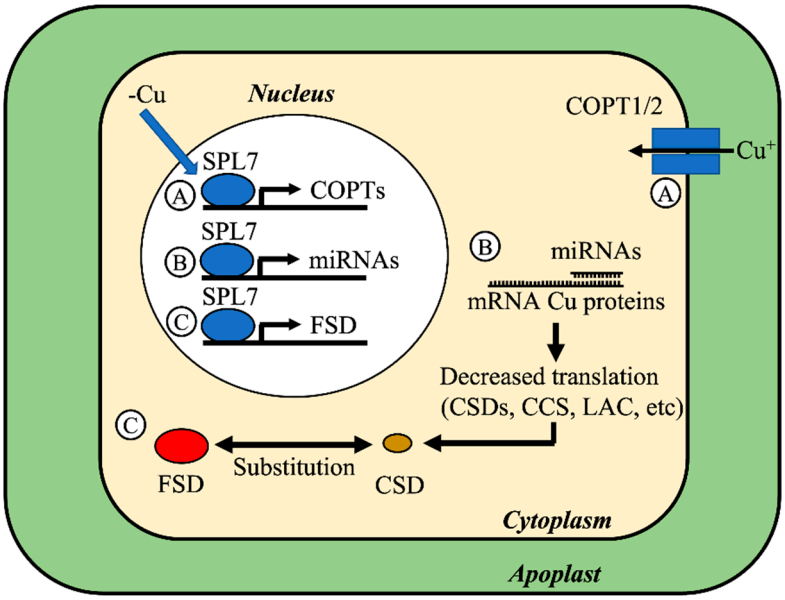

Figure 1. The Cu economy response in *Arabidopsis thaliana* is orchestrated by AtSPL7. A Cu deficiency signal activates AtSPL7, which in turn binds to CuRE (Cu Responsive Elements) in target gene promoters. The AtSPL7 targets are: (**A**) high affinity Cu transporters from the COPT family, which are targeted to the plasma membrane and promote Cu uptake from the extracellular space; (**B**) miRNAs from the miRNA397, miRNA398, miRNA408, and miRNA827 families, which target mRNAs from proteins that use Cu as cofactor, such as Cu/ZnSOD (CSDs), Laccases (LAC), and Cu chaperones (such as CCS), decreasing the Cu usage and saving Cu for plastocyanin; (**C**) proteins that perform similar functions as Cu-using counterparts, such as FeSOD (FSD), which accumulate and substitute cuproproteins such as CSDs.

4.2. Copper Uptake from the Soil

Copper can adopt oxidation states Cu^{2+} and Cu^{+}, making it biologically useful for single electron transfers. In the soil, Cu^{2+} is typically the most abundant form, while uptake by *A. thaliana* roots occurs as Cu^{+}, suggesting that Cu reduction is necessary at the rhizosphere. In one of the first works describing the transcriptional regulation of Cu deficiency that depends on AtSPL7, two proteins, namely AtFRO4 and AtFRO5, were shown to be up-regulated in a AtSPL7-dependent manner under Cu deficiency, and to function as Cu^{2+} reductases at the root surface (Figure 2). *AtFRO4* and *AtFRO5* genes are located in tandem in the *A. thaliana* genome, and proteins encoded by these genes are necessary for Cu uptake, including them in Cu deficiency regulon controlled by AtSPL7 [107]. This resembles the Fe uptake mechanism found in *A. thaliana* roots, which also depends upon an Fe^{3+} reductase (AtFRO2) for reduction before uptake by Fe^{2+} high-affinity transporter AtIRT1 [113]. AtFRO2 is part of the same gene family as AtFRO4/AtFRO5, as well as other metal homeostasis-related reductases [114]. Interestingly, while there are eight *FRO* genes in the *A. thaliana* genome, only two are present in rice [115], and none was linked to root surface reduction of either Fe or Cu. Therefore, rice plants might rely on distinct mechanisms for Cu uptake, as already shown for Fe [116]. In fact, participation of FRO proteins in Cu uptake in other plant species is still lacking, making it an interesting avenue for future research.

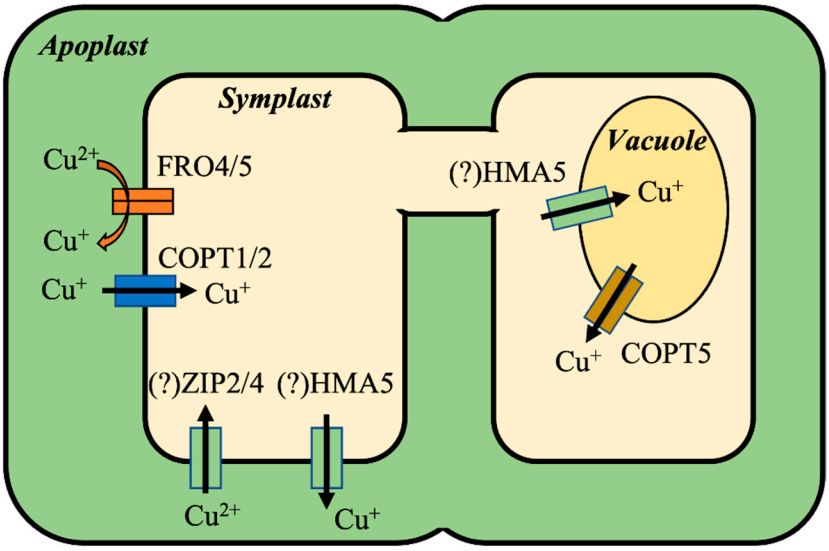

Figure 2. Representation of *Arabidopsis thaliana* Cu uptake and vacuolar Cu transport in a root cell. Cu^{2+} is reduced to Cu^{+} by extracellular, membrane-bound reductases AtFRO4/AtFRO5 in the rhizosphere. Cu^{+} is transported into root cells by high affinity transporters AtCOPT1 and AtCOPT2. Possible uptake of Cu^{2+} by AtZIP2 and/or AtZIP4 is also represented. AtHMA5 Cu detoxification by either vacuolar internalization or plasma membrane efflux is shown. AtCOPT5, which is located in both pre-vacuolar compartments and tonoplast, is involved in Cu transport from the vacuole to the cytoplasm. Two generic cells connected by plasmodesmata are represented to accommodate depictions of both plasma membrane and vacuolar transporters.

4.3. COPT Transporters in Arabidopsis thaliana

After reduction by FRO proteins, Cu^{+} is transported into root cells by high-affinity transporters of the COPT (Copper Transporters) family. COPTs are described in several plant species [117–119]. The X-ray structure of the homologous protein CTR1 from Atlantic salmon (*Salmo salar*) shows that COPT proteins, which harbor three transmembrane domains, function as a homotrimer. The authors also showed two methionine triads in the second transmembrane domain acting as selectivity filters for Cu uptake [120]. Interestingly, yeast two hybrid evidence suggest that COPT proteins from rice can interact each other [119], raising the possibility that heterotrimers might be functional. However, this hypothesis needs to be thoroughly tested.

The *A. thaliana* genome has six COPT members. AtCOPT4 is the only member from the gene family in *A. thaliana* that could not complement yeast mutants deficient in Cu uptake, and therefore, is considered nonfunctional [117]. AtCOPT1, AtCOPT2, and AtCOPT6 are up-regulated by Cu deficiency and localized in the plasma membrane (Figure 2; [4,117,121–123]). AtCOPT1 is the main plasma-membrane-localized Cu transporter involved in Cu uptake at root tips, and is regulated by AtSPL7 (Figure 1; [107,123,124]). In a detailed study using *A. thaliana* plants stably expressing GFP-tagged AtCOPT1 proteins, AtCOPT1 was shown to localize at both plasma membrane and endoplasmic reticulum (ER). Interestingly, authors showed that AtCOPT1 is degraded upon Cu excess exposure by the 26S proteasome, but independently from ubiquitination [125]. Another recent work has shown that AtCOPT1 interacts with two UBIQUITIN-ASSOCIATED DOMAIN-CONTAINING PROTEIN 2 (UBAC2), localized in the ER and involved in COPT stabilization and protection against degradation by 26S proteasome. Under Cu deficiency, UBAC2's promotes AtCOPT1 stabilization at the ER before secretion to the plasma membrane, which in turn results in Cu accumulation, demonstrating a new and complex mechanism to control Cu uptake in plants. UBAC2's interact with AtCOPT2 and AtCOPT6 as well, although these interactions were not extensively detailed yet [126]. Considering that these proteins can function as dimers, it would be interesting to test whether UBAC2 proteins might be stabilizing the heterodimers in vivo. Taken together, these works showed for the first time

that COPTs have multiple levels of regulation in order to acclimate plants to varying Cu levels in the soil.

AtCOPT2 is located at the plasma membrane and ER, is up-regulated by Cu deficiency in an AtSPL7-dependent manner, and is expressed in roots (Figure 1). However, it seems to constitute a secondary pathway for Cu uptake, as it is expressed in distinct domains compared to AtCOPT1 [122,127]. Similar to AtCOPT1, AtCOPT2 is also degraded by Cu excess through 26S proteasome independently from ubiquitination. The authors suggest that upon Cu excess, AtCOPT2 might be diverted from plasma membrane targeting to ER-associated proteasome degradation [127]. Since this is reminiscent of AtCOPT1 [125], it is possible that both proteins are post-translationally regulated by a similar, concerted mechanism. Moreover, AtCOPT2 is also induced by Fe deficiency and is implicated in delivering Cu to multicopper oxidases LPR1 and LPR2, which are described as important for root low phosphorus (P) response. Therefore, AtCOPT2 might link P, Fe, and Cu responses in *A. thaliana* roots [4,121,122].

AtCOPT6 is also localized to the plasma membrane, up-regulated by low Cu concentration, and is expressed mainly in reproductive and aerial vascular tissues, including stems, leaves, meristems, stomata, trichomes, filaments, and stamen in pre-anthesis flowers, on pollen in post-anthesis flowers, and seed envelope and embryo [128,129]. Interestingly, AtCOPT6 was shown to interact with AtCOPT1 and with itself [128]. AtCOPT6 is proposed to function in adequate Cu distribution in *A. thaliana* shoots under Cu deficiency, since *atcopt7* mutant plants show increased Cu concentration in rosettes but decreased in seeds when grown under Cu deficiency. Together with the observed expression pattern, this result suggest that AtCOPT6 is involved in Cu delivery to filial tissues in seeds [129]. Moreover, it seems that AtSPL7 is at least partially involved in the transcriptional control of AtCOPT6 expression in aerial tissues in response to Cu fluctuations [128].

AtCOPT3 and AtCOPT5 are not regulated by Cu concentration, are localized in internal membranes, and are involved in intracellular Cu remobilization [130–132]. *AtCOPT5* is expressed mainly in vasculature and roots. While the protein localizes to internal membranes, one study showed localization to the pre-vacuolar compartment [130], while another suggested vacuolar localization (Figure 2; Klaumann et al. 2011 [131]). Although its precise function is not yet clear, it is likely involved in Cu remobilization from pre-vacuolar vesicles/vacuole to the cytoplasm under Cu deficiency, acting as a Cu exporter, since vacuoles of *atcopt5* mutants show increased Cu concentration [130,131]. Moreover, *AtCOPT5* loss-of-function results in increased Cu concentration in roots, and decreased in seeds, suggesting that lack of a Cu exporter decreased root-to-shoot translocation, and implicating AtCOPT5 in Cu long distance transport [131].

AtCOPT3 is localized to compartments of the secretory pathway and expressed in the leaf vasculature, anthers, and pollen grains [132]. AtCOPT3 is clearly not involved in direct Cu uptake from the soil, since it has low expression in roots. However, it was shown to be important for daily fluctuation in Cu homeostasis. Some TCP proteins interact with key clock proteins in *A. thaliana*, linking these proteins to circadian rhythm [133]. Interestingly, *AtCOPT3* was shown to be regulated by AtTCP16 (named after TEOSINTE BRANCHED 1, CYCLOIDEA, and PROLIFERATING CELL FACTOR 1) transcription factor, which is known to function in pollen development [134,135]. Plants with increased expression of *AtTCP16* were more sensitive to Cu deficiency, while AtTCP16 was shown to bind to AtCOPT3 proximal promoter (as well as to *AtCOPT5* promoter, which was not further explored) to repress its expression, with *AtTCP16* expression oscillating diurnally, which is opposite to *AtCOPT3*. *AtCOPT3* is also repressed by excess Cu, which is at least partially regulated by AtTCP16. Moreover, *atcopt3* mutants showed a higher percentage of pollen ornamentation defects when grown under Cu deficiency, in agreement with AtCOPT3 expression in reproductive organs [132]. Taken together, the results indicate that AtTCP16 is a repressor of AtCOPT3 in circadian oscillation as well as in response to internal Cu levels.

Finally, it is noteworthy that *AtCOPT1* and *AtCOPT3* are located head-to-head in the genome, and likely to share a bidirectional promoter, since *AtCOPT1* is also expressed in pollen development [136]. Cu is required for proper pollen development, and both bHLH family members, SPL7, and Cu-DEFICIENCY-INDUCED TRANSCRIPTION FACTOR 1 (CITF1, also named bHLH160), were demonstrated to have a function in Cu transport to anthers [137], suggesting that exploring the role of such transporters in reproductive development is promising.

4.4. COPT Proteins Characterized in Other Species

COPTs were also characterized in other species, although to a much lower extent. In rice, there are seven COPT genes. Plasma membrane-localized OsCOPT1 and OsCOPT5 were the first two family members characterized [138]. Interestingly, it was shown that the two transporters function by physically interacting the transmembrane protein Xa13, which is part of the poorly characterized MtN3/saliva family. Xa13 is induced upon infection by certain strains of *Xanthomonas oryzae* (Xoo), which can cause bacteria blight disease by spreading within the xylem vessels of rice shoots [139]. It was shown that one pathogenic Xoo strain induces not only *Xa13*, but also *OsCOPT1* and *OsCOPT5*, and that the three proteins interact each other and are necessary to reduce Cu concentration in xylem sap, which in turn favors Xoo survival. Plants over-expressing either *Xa13*, *OsCOPT1*, and *OsCOPT5* showed increased susceptibility, decreased Cu concentration in xylem sap, and increased Cu concentration in shoots. Altogether, the authors were able to characterize two Cu transporters in rice, showing that they play a role in the plant–pathogen interaction [138].

OsCOPT1 and OsCOPT5 interact each other and with themselves [138]. In an overall characterization of the other rice COPT genes, it was shown that self-interaction is a common feature [119]. The authors also showed that OsCOPT2, OsCOPT3, and OsCOPT4 interact with OsCOPT6, suggesting that homo- and heterotrimeric COPT complexes can be formed to facilitate Cu transport in rice. *OsCOPT1*, *OsCOPT5*, and *OsCOPT7* are up-regulated by Cu deficiency and down-regulated by Cu excess in roots and shoots [112,119,138], while *OsCOPT6* is regulated similarly but only in shoots, with no detectable expression in roots. *OsCOPT2*, *OsCOPT3*, and *OsCOPT4* are down-regulated by Cu excess, but no change is observed in response to Cu deficiency [119].

A few examples of COPT functional characterization are available for other plant species. *Medicago truncatula* MtCOPT1 was shown to be a Cu transporter localized to the plasma membrane and expressed only in association with nodules. Plants lacking *MtCOPT1* have reduced biomass and nitrogenase activity. Since nitrogenase depends on other metals such as Fe and Mo but not Cu, the observed result is likely due to reduced Cu-dependent functions such as cytochrome oxidase, which utilizes free O_2 and decreases nitrogenase poisoning [140]. Moreover, two *Physcomitrella patens* COPTs were also characterized as Cu plasma membrane transporters, which are up-regulated by Cu deficiency and down-regulated by Cu excess [141], which suggest that using COPTs as high-affinity Cu transport from the soil is ancient in land plants.

4.5. A Possible Role of ZIPs in Copper Homeostasis

Early works in *A. thaliana* have proposed that two proteins from the ZIP (Zinc-regulated/Iron-regulated Protein) transporter family, AtZIP2 and AtZIP4, could use Cu as a substrate [142]. Both genes are regulated by Cu availability in *A. thaliana*, with AtZIP2 being up-regulated, although there are some contradictory results in the literature regarding its regulation by Cu deficiency and excess [101,107,142,143]. In any case, it is still an open question whether AtZIP2/AtZIP4 are involved in Cu root uptake (Figure 2). A similar regulation at the transcriptional level was observed in orthologous genes in *Vitis vinifera* [144], which suggests these ZIP transporters might have a role in Cu homeostasis. It is important to highlight that the ability to transport Cu is still lacking confirmation, as

no ZIP transporter was shown to use Cu as a substrate to date. Therefore, the physiological role of this gene family in Cu homeostasis is still unclear.

4.6. PIB-Type ATPase/Heavy Metal-Associated (HMA) Proteins and Their Role in Copper Homeostasis in Arabidopsis thaliana

P_{IB}-type ATPase are proteins that couple cation transport with ATP hydrolysis. They are ubiquitous in the tree of life, comprising a large family of proteins in plants, with 6–8 transmembrane domains and a Heavy Metal-Associated domain [145]. Besides Cu, HMA transporters might also transport other metals such as Zn and Cd [146–148]. Still, a subset of HMAs use Cu as substrate, with roles in either Cu efflux from cells when located at the plasma membrane or detoxification into vacuoles when in the tonoplast [4].

In *A. thaliana*, AtHMA5 was shown to be induced by Cu excess in roots, and loss-of-function *hma5* mutants are more sensitive to Cu, although accumulate higher Cu concentration in roots. However, the subcellular localization of AtHMA5 is not clearly determined. Therefore, its proposed role is Cu detoxification, either by efflux from the cytosol or compartmentalization into the vacuole (Figure 2; [149]). AtHMA5 is also linked to Cu tolerance natural variation in *A. thaliana* ecotypes, since those containing amino acid substitution that results in weak/hypofunctional alleles show higher sensitivity to excess Cu [150].

In chloroplasts, Cu is essential, being a cofactor of both plastocyanin and Cu/Zn SOD. Two HMA transporters are key for Cu deliver from the cytoplasm into chloroplasts, namely AtHMA6 and AtHMA8 (Figure 3). Mutant plants for *AtHMA6* (also known as PAA1, or P-type ATPase in Arabidopsis) show decreased Cu concentration in chloroplasts, although the overall concentration in leaves is not modified. *athma6* mutants also show Cu-dependent defects in electron transport, as well as reduced Cu/Zn SOD activity [151]. AtHMA6 is localized to the periphery of chloroplast envelope, which suggests a function in transporting Cu from the cytoplasm into the stroma [152]. Interestingly, AtHMA8 (also known as PAA2) is also involved with Cu transport in chloroplasts, but is localized to the thylakoid membrane. Consistently, *athma8* shows decreased Cu delivery to plastocyanin, but Cu in the stroma is not reduced, suggesting the two transporters function in tandem: AtHMA6 moves Cu from the cytoplasm to the stroma, and AtHMA8 from the stroma to the thylakoid lumen (Figure 3; [152,153]). This sequential Cu transport system seems to have evolved early in the plant lineage [153].

Another P_{1B}-type ATPase localized to the chloroplast envelope was described in *A. thaliana*. AtHMA1 is expressed in green tissues, and *athma1* plants show decreased SOD activity, but normal plastocyanin function, which is consistent with Cu transport into the chloroplast stroma, but not to the thylakoid lumen (Figure 3). Mutant plants are also more sensitive to high light, suggesting that AtHMA1 function similarly to AtHMA6, but could be more important for growth under adverse light conditions [154].

An unexpected link between ethylene signaling and Cu homeostasis was established. The ethylene receptor ETR1 has Cu as cofactor, which is necessary for ethylene binding [155]. The P-type ATPase AtHMA7 was shown to be necessary for ETR1 biogenesis and function, since it provides Cu ions for the receptor [156]. Interestingly, the Cu chaperone AtATX1 (see section below) was shown to provide Cu ions to AtHMA7, which in turn delivers them for ETR1 biogenesis [157]. However, there is still much to discovery regarding AtHMA7 and its role in Cu homeostasis, and these points should be addressed by future studies.

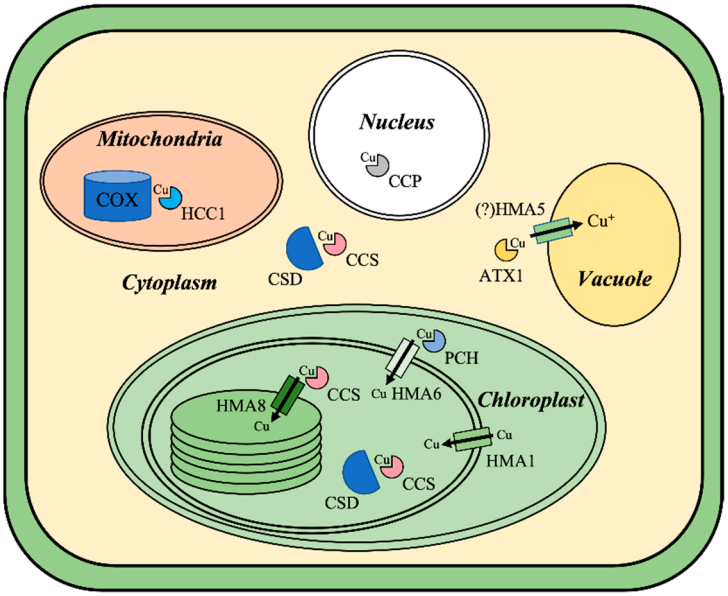

Figure 3. Copper chaperones in *Arabidopsis thaliana* and their roles in Cu homeostasis. An *A. thaliana* cell is represented with all organelles for which Cu chaperones are described. In the chloroplast, inner envelope and thylakoid membrane transporters are shown. AtPCH is the Cu chaperone to AtHMA6, and AtCCS is chaperone to AtHMA8, as well as to stroma localized CuZnSOD (CSD). AtCCS is the chaperone to CSD localized in the cytoplasm. AtATX1 is shown as the Cu chaperone to AtHMA5 (for simplification, AtHMA5 is shown only in the tonoplast but not at the plasma membrane). AtHCC1 is the Cu chaperone likely delivering Cu to mitochondrial COX complex. AtCCP is a Cu chaperone important for plant immunity which localizes to the nucleus, but its interactions and specific role in Cu homeostasis is still unknown.

4.7. HMA Copper Transporters in Other Species

The first HMA protein described in monocots was OsHMA9 [158]. OsHMA9 is expressed in vascular bundles and anthers of rice plants, and is up-regulated when plants are exposed to high Cu concentration. Localized to the plasma membrane, it can efflux Cu from yeast cells, and *oshma9* mutant plants show sensitivity to high Cu, suggesting that OsHMA9 is important for Cu detoxification [158].

The rice protein OsHMA5 was shown to be involved in Cu xylem loading and root-to-shoot Cu translocation. OsHMA5 is localized at the plasma membrane, is induced by Cu excess, and is expressed in root perycicle at the vegetative stage, but in nodes and reproductive organs at the reproductive stage. Loss-of-function *oshma5* mutants show decreased Cu concentration in shoots and seeds, but increased Cu concentration in roots [159]. Recently, *oshma5* mutants were used as a tool to identify genes participating of the Cu economy response in rice, since *oshma5* shoots are likely more prone to Cu deficiency than WT ones, while the roots showed an opposite behavior. The authors identified genes that are likely involved in Cu economy, which seems to be partially conserved when compared to *A. thaliana* (please see section above; [112]).

OsHMA4 was described in rice as a tonoplast-localized Cu transporter involved in Cu sequestration within the root vacuoles. Mutant plants *oshma4* show decreased Cu concentration in aerial tissues, including shoots and grains. Interestingly, OsHMA4 alleles were associated with variation in Cu concentration in diversity panels, linked to single amino acid substitution, which leads to hypofunctionality [160]. Interestingly, a similar gene performing OsHMA4 function was not described in *A. thaliana* or other plants, raising the question as to whether Cu vacuolar sequestration is performed by unrelated transporters in other species [161].

4.8. Copper Chaperones in Model Species

Copper can be noxious when accumulated inside the cells, and therefore, the amount of free Cu is probably less than one free ion per cell [162]. To move within the cells, Cu are bound to chaperones, which avoid toxicity and deliver Cu to other proteins [163,164]. Early work in *A. thaliana* described two Cu chaperones, named Antioxidant Protein1 (ATX1) and ATX1-Like Copper Chaperone (CCH). AtHMA5 can physically interact with ATX1 (Figure 3), which is thought to be important for Cu deliver to AtHMA5 [149]. Plants lacking ATX1 function show similar phenotypes to *athma5*, such as hypersensitivity to both Cu excess and deficiency. Overexpression of ATX1, on the other hand, increases plant tolerance to Cu excess [165]. These results establish a role for the Cu chaperone ATX1 in Cu homeostasis, likely through Cu deliver to AtHMA5. The role of CCH, however, is still not clear.

A similar gene to *AtATX1* was described in rice plants. OsATX1 interacts with OsHMA4, OsHMA5, OsHMA6, and OsHMA9, likely delivering Cu to these P-type ATPases. OsATX1 seems to have a function in Cu distribution in rice plants, since its overexpression results in reduced Cu concentration in roots, but increased Cu concentration in shoots; *osatx1* mutant plants, on the other hand, show increased Cu concentration in roots. Interestingly, the Cu concentration in reproductive tissues followed a similar pattern (increase in overexpressing plants and decrease in mutant plants) [166].

Another interesting Cu chaperone was described for AtHMA6, the chloroplast inner envelope transporter (Figure 3). Named AtPCH1 (Plastid Chaperone 1), the protein is derived from an alternative splicing event of *AtHMA6* gene in several (but not all) plant species, including algae but not cyanobacteria. AtPCH1 is encoded by a different locus according to the species, being independently originated by duplication and subsequent subfunctionalization in plant lineages within Poaceae and Brassicaceae. Interestingly, authors also showed that the Cu/Zn SOD chaperone CCS can interact with AtHMA8 (Figure 3), providing evidence that chaperones deliver Cu to each transporter involved in Cu uptake into the thylakoid lumen [153].

The Copper Chaperone for Superoxide dismutase (CCS) is the protein responsible for Cu delivery to Cu/ZnSOD (Figure 3; Chu et al. 2005 [167]). Interestingly, Cu/ZnSOD activity is detected in the cytosol, chloroplast, and peroxisome, encoded by different *SOD* genes, while only one *AtCCS* gene is found in the *A. thaliana* genome [167,168]. Data suggest that Cu/ZnSOD can be activated via CCS-dependent and CCS-independent pathways, the latter involving glutathione [169]. This varied according to SOD isoform and its location: cytoplasm (CCS-dependent and independent activation), chloroplast (CCS-dependent only), and peroxisome (CCS-independent only). In any case, the same CCS protein is the SOD Cu chaperone in both cytoplasm and chloroplast [167,168]. Moreover, the *CCS* transcript is a target for the Cu deficiency-induced miR398 family, which down-regulates Cu/ZnSODs as well [168], suggesting that decreasing Cu deliver to SOD is part of the Cu economy response.

Cytochrome c oxidase (COX) is a protein complex that is essential for mitochondrial aerobic metabolism. COX assembly involved many proteins, and includes a CuA site at CoxII protein, which has two Cu, and another CuB site with one Cu [170,171]. Two Cu chaperone duplicated genes, named *AtHCC1* (Homologue of Copper Chaperone SCO1; SCO1 being the yeast homologous gene) and *AtHCC2*, were considered possible chaperones for COX complexes [172,173]. Both proteins are found in the mitochondria. AtHCC1 was shown to be essential for COX activity and plant survival (Figure 3), with loss-of-function mutants being embryo-lethal. AtHCC2, on the other hand, does not seem to be related to COX assembly, since *athcc2* mutants retain COX activity, but instead show a UV-B sensitive phenotype [174]. Later, a work using plants knocked down for either *AtHCC1* or *AtHCC2* showed that while plants with low *AtHCC1* expression showed more sensitivity to salt stress, plants low in *AtHCC2* showed the opposite phenotype. This was followed by other biochemical and molecular markers, suggesting that AtHCC1 is important for COX

assembly and activity, whereas AtHCC2 seems to negatively modulate AtHCC1 by directly interacting with it [175].

Recently, a new Cu chaperone was described in *A. thaliana*. Curiously, this protein (AtCCP) is induced by pathogens (Figure 3; Copper Chaperon induced by Pathogens). Besides a typical Cu binding site, AtCCP also harbors a NLS (Nuclear Localization Signal), which is necessary for its nuclear localization. Overexpression of *AtCCP* results in increased expression of *PR* genes and higher tolerance to *Pseudomonas syringae* infection, whereas loss-of-function mutants showed impaired plant immunity, suggesting that AtCCP is necessary for plant response to pathogens and involved in the salicylic acid pathway [176].

4.9. The Role of Yellow Stripe-like (YSL) Transporters in Copper Homeostasis

The Yellow Stripe-like (YSL) family of transporters is mostly involved in Fe and Mn transport, as these proteins transport metals bound to phytosiderophores and nicotianamine (NA) [177–180]. Especially in Poaceae species, such as maize (*Zea mays*), barley (*Hordeum vulgare*), rice and its more closely related wild species (the *Oryza* complex), YSL proteins are involved in the uptake of Fe^{3+}-phytosiderophore complexes from the soil, which is a key component of the Strategy II or chelation strategy [116,181,182]. *YSL* genes are present in all plant genomes sequenced to date, and the encoded proteins have functions in metal distribution within the plant. However, only a few YSL proteins were shown to transport Cu.

In *A. thaliana*, AtYSL1 and AtYSL3 were shown to be plasma membrane transporters of metal-NA complexes. Despite evidence showing that both are Fe-NA transporters, plants lacking both transporters have disturbed Cu distribution, with increased and decreased Cu concentration in leaves and seeds, respectively [180,183]. AtYSL2, which is closely related to AtYSL1 and AtYSL3, was shown to transport Cu-NA complexes [184], although other authors working with the same protein did not find the same results [185]. Therefore, although it seems clear that YSL proteins in *A. thaliana* have at least an indirect role in Cu homeostasis [186], it is not yet clear whether they can transport Cu and what are their physiological functions regarding Cu distribution.

In rice, however, OsYSL16 was unequivocally shown to transport Cu-NA [187,188]. *OsYSL16* is expressed in roots, leaves, and nodes, with higher expression in nodes during reproductive stage, and especially in the phloem of the node and vascular tissues of the leaves. *osysl16* loss-of-function plants showed increased Cu concentration in older leaves, but decreased Cu concentration in younger leaves, suggesting a defect in Cu mobilization from older to younger leaves. Plants lacking OsYSL16 function also showed reduced Cu concentration in seeds, and reduced fertility [187]. Expression in the vascular bundles of rachilla was shown to be important for adequate Cu delivery to stamens, explaining the low fertility in mutant plants, while expression in palea and lemma vasculature showed that OsYSL16 is key for redistribution of Cu in seeds [188].

Recently, *OsYSL16* closest homolog *BdYSL3* in *Brachypodium distachyon* was functionally characterized. *B. distachyon* is a model species closely related to wheat (*Triticum aestivum*), and useful to understand Cu homeostasis in cereal crops [189]. It was shown that both wheat and *B. distachyon* have decreased fertility and altered flower development when exposed to Cu deficiency. BdYSL3 localizes to the plasma membrane, transport Cu in *Xenopus laevis* oocytes, and is expressed in the phloem. Loss-of-function of *BdYSL3* results in decreased Cu delivery to reproductive organs, resulting in delayed flowering, changes in inflorescence architecture, lower seed set, and decreased seed weight and size. These results demonstrate the importance of Cu in reproductive development, and how low Cu levels can affect crop productivity [190].

5. Iron and Copper Crosstalk

5.1. Iron and Copper Crosstalk in Arabidopsis thaliana

Interactions of micronutrients can affect the absorption and the bioavailability of other nutrients by a number of mechanisms that remain unclear [12]. The crosstalk between Cu

and Fe homeostasis have been previously documented in humans, yeast, and green algae, but it is not well elucidated in plants [191–193]. Metal homeostasis is mostly studied for each isolated element; however, metal interactions cannot be predicted from the response to individual elements. Given that Cu and Fe interaction seems to be particularly important for understanding Cu homeostasis, we specifically review the current knowledge on this interaction here.

Like Cu, Fe is also essential for plant growth and development, but can be toxic when in excess. Therefore, plants regulate uptake, absorption, and intracellular transport of Fe ions in cells [113]. Although the molecular details of the crosstalk between Fe and Cu remain to be elucidated, the amount of evidence showing the occurrence of this interaction has increased in the last ten years [107,194–198].

One of the earliest interactions described was in plants under Cu deficiency and the Cu economy response, when Arabidopsis cytosolic (AtCSD1) and stromal (AtCSD2) forms of Cu/ZnSOD metalloproteins are replaced by the Fe counterparts FeSODs, aiming to save Cu for essential cuproproteins as plastocyanin (please see the section above) [4,15,108,110,199]. This mechanism is under control of the transcription factor AtSPL7, and its downstream target miR398 [101,107]. Conversely, Fe deficiency leads to replacement of FeSODs for Cu/ZnSODs by down-regulation of miR398 and up-regulation of *AtCSD1* and *AtCSD2* genes under low Fe [194]. Recent work suggests that such mechanism is at least partially conserved in rice [112].

AtCOPT2, a member of the COPT family of transporters in *A. thaliana*, is a plasma membrane protein which plays a role on high-affinity Cu acquisition and Cu distribution. The expression of *AtCOPT2* is induced by both Cu and Fe deficiencies [122]. In response to Fe deficiency, the COPT2 regulation partially depends on FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) [200,201]. Additionally, the characterization of knockout *copt2-1* lines showed improved resistance to simultaneous Fe and Cu deficiencies, by maintenance of the photosynthetic apparatus and improved plant growth and seed production [122]. Furthermore, when plants are cultivated under Cu excess, the induction of *AtCOPT2* in roots by Fe deficiency is abolished, which suggests a role for Cu in FIT-regulation responses in *A. thaliana* roots, and that *AtCOPT2* plays a role in the attenuation of Cu deficiency responses driven by Fe deficiency possibly to avoid further Fe consumption [122]. Additionally, a significant percentage of induced genes in *atcopt2* lines under Fe and Cu deficiencies is related to the phosphate (P) starvation response, indicating an effect of *AtCOPT2* also in P regulation, and the interaction of Fe, Cu, and P homeostasis [122].

The proteins AtCOPT1 and AtCOPT3 are located into the plasma membrane and at a compartment of the secretory pathway, respectively [123,132]. Recently, comparative transcriptomic analysis of seven-day-old WT and OE-*AtCOPT1* plants under Cu deficiency and mild Cu excess conditions showed that Fe homeostasis is affected in OE-*AtCOPT1* plants under both conditions. OE-*AtCOPT3* plants showed lower *AtIRT1* expression than WT under both non optimal Cu supply, as observed for OE-*AtCOPT1* plants [198]. Furthermore, OE lines had slightly lower Fe concentration than the WT in both shoots and roots when cultivated under Cu sufficiency or excess, and the decrease in Fe concentration was more evident in roots of the OE-*AtCOPT3* seedlings, suggesting that unregulated Cu uptake under high Cu repressed Fe uptake mechanisms, probably due to the down-regulation of FIT protein. Additionally, an increased Fe^{3+}/Fe^{2+} ratio in OE-COPT roots was observed, which probably disturbs the signaling pathways and local responses to Fe deficiency, consequently inhibiting the Fe uptake [198]. However, when OE-*AtCOPT1* plants were cultivated under moderated Fe deficiency and Cu sufficiency, expression of both *AtIRT1* and *AtFIT* increased, suggesting that OE-COPT seedlings responded as being in Fe deficiency when under Cu sufficiency, and the alterations in Fe homeostasis were restricted to Cu deficiency and excess [198].

atspl7 mutants cultivated in Cu-deficient media showed increased expression of Fe-deficiency markers *AtIRT1* and *AtFRO2*, whereas catalase activity and Ferritin1 protein

levels were decreased, indicating that Cu deficiency leads to Fe deficiency responses in shoots. Impairment of root-to-shoot Fe translocation in *atspl7* mutants was observed, corroborating the reduction on total shoot Fe concentration observed in this line when grown under Cu deficiency compared with WT [107], reinforcing the idea that an adequate Cu nutritional status is required to maintain Fe partitioning in *A. thaliana*. Interestingly, *atspl7* mutants mostly restored growth defects when cultivated under Fe deficiency, which in turn increase Cu concentration in shoots. Furthermore, genes classically responsive to Fe stress, as *AtFRO2*, *AtIRT1*, *AtMYB10*, *AtMYB72*, *AtNAS4*, and *AtIMA3* were induced in roots of *atspl7* mutant in response to Fe deficiency, indicating that the response to Fe deficiency is not affected. Similarly, genes encoding transcription factors from subgroup Ib bHLHs; *AtbHLH38*, *AtbHLH39*, *AtbHLH100*, and *AtbHLH101*, *AtFRO3*, and all *IMA* genes were up-regulated in rosettes under Fe deficiency. In addition, an induction of *AtCOPT2* and *AtFRO4* in roots of *atspl7* mutants was also observed, suggesting that plants were experiencing simultaneous Fe and Cu deficiency. These results reinforce that both *AtFIT* and *AtSPL7* contribute to Fe-Cu crosstalk [202], and if Cu or Fe uptake is hindered, plants accumulate excessive concentrations of the other metal.

AtCOPT5 is a pre-vacuolar/vacuolar compartment transporter which plays an important role in the plant response to environmental Cu deficiency by remobilizing Cu from root vacuoles and from roots to reproductive tissues under Cu deficiency stress (see previous sections; Klaumann et al. 2011 [131]; Garcia-Molina et al. 2011 [130]). NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN3 and 4 (NRAMP3 and NRAMP4) transporters redundantly function in Fe mobilization from vacuoles in seeds during germination and in adult plants [203,204]. A recent study showed that AtCOPT5 participates in the crosstalk between vacuolar Fe and Cu pools mobilization [197]. In *atcopt5* mutants under Cu deficiency, the expression of Fe-related genes (*AtIRT1*, *AtYSL1*, *AtBTS*, *AtbHLH38*, *AtbHLH39*, *AtbHLH100*, *AtbHLH101*, and *AtNRAMP4*) was induced. *AtCOPT5* loss-of-function affects Fe localization and several Fe deficiency responses, as mutant plants were more sensitive to Fe deficiency than the WT ones [197]. As both AtNRAMP4 and AtCOPT5 are located in the tonoplast, and *AtNRAMP4* is induced by Cu deficiency in *atcopt5* mutants, the double mutant *nramp3nramp4* was cultivated under mild and severe Cu deficiency. *nramp3nramp4* lines were found to be highly sensitive to Cu deficiency compared to the WT, and the *AtCOPT5* expression was induced in this line, suggesting enhanced COPT5-dependent Cu remobilization from the vacuoles. Additionally, a connection between the vacuolar Cu transport and Fe distribution was suggested as lower Fe in seedlings was observed in the *atcopt5* mutants compared to the WT, and the seeds from *copt5* showed enhanced Fe concentration, suggesting a role for *AtCOPT5* in Cu-dependent Fe mobilization during the germination process [197]. Interestingly, the *nramp3nramp4* mutant showed an opposite pattern when Cu and Fe concentrations were evaluated. These results suggest that the Fe and Cu vacuolar pools are interconnected, since the loss-of-function of one tonoplast metal transporter for one of the metals drives the remobilization of the other by inducing the expression of the corresponding vacuolar transporter [197].

Under Fe deficiency, *A. thaliana* employ the strategy I for Fe uptake, which involves several bHLH TFs (for a detailed review, please check Brumbarova et al. 2015 [205], Schwarz and Bauer 2020 [206], Riaz and Guerinot 2021 [113]). Cai et al. (2021) [207] observed that Cu-responsive genes *AtFRO4*, *AtFRO5*, *AtLAC2*, *AtLAC7*, *AtLAC12*, and *AtCOPT2* are also up-regulated in response to Fe deficiency. However, up-regulation of *AtCOPT2*, *AtFRO4*, and *AtFRO5* in *atfit* or in a quadruple loss-of-function mutant lines of *atbhlh38*, *atbhlh39*, *atbhlh100*, and *atbhlh101* (*bhlh4x*) was blocked under Fe-deficiency conditions, suggesting that both AtFIT and bHLHs TFs mutually promote the expression of Cu uptake genes and mediate the crosstalk between Fe and Cu homeostasis. Both mutant lines, *atfit* and *bhlh4x*, also showed a reduction in Cu concentration, when compared with WT, under Fe deficiency conditions. On the other hand, co-overexpression lines from *AtFIT* and *AtbHLH38* and *AtbHLH39* (OE-*AtFIT*/OE-*AtbHLH38*, OE-*AtFIT*/OE-*AtbHLH39*) accumulated more Cu than the WT independently from the Fe status, suggesting that

AtFIT and bHLH Ib promote the Cu uptake in response to Fe deficiency [207]. Therefore, *AtFRO4*, *AtFRO5*, and *AtCOPT2* are SPL7-dependent under Cu deficiency [107]; however, the expression of these genes is not dependent on SPL7, but dependent of FIT and bHLH 1b TF under Fe deficiency. Yet, increased Cu concentration under Fe deficiency is dependent on both AtFIT and AtSPL7 [207].

Laccases (LAC) are glycoproteins which belong to a subgroup of multicopper oxidases (MCOs), able to catalyze the oxidation in vitro of a wide variety of phenolic, inorganic, and/or aromatic amine substrates [208–210]. More recently, *miR408* was described to participate antagonistically in the response to low Cu and Fe supply [211,212]. As observed for *miR398*, *miR408* is also up-regulated by AtSPL7 under Cu deficiency [213]. miR408 targets the mRNAs of laccase genes [108]. *AtLAC12* is also up-regulated by Fe deficiency conditions [214]. Additionally, *atlac12* mutant lines were more sensitive to Fe deficiency than WT seedlings and root Fe concentration was higher in *atlac12* mutant line, while shoot Fe concentration was lower, suggesting a role of *AtLAC12* in root-to-shoot Fe partitioning, under Fe limited conditions. On the other hand, Cu concentrations increased in roots and shoots of *atlac12* mutants under Fe deficiency, when compared to control [214].

Besides miR398, miR408 is also antagonistically regulated by Cu and Fe deficiencies [211], and is required for proper vegetative development of *A. thaliana* plants, being associated with adaptation to different abiotic stresses [105,213,215]. The regulation of miR408 under Cu deficiency is mediated by AtSPL7 [107,213]. Previous experiments employing knockout and OE lines showed that appropriate expression of miR408 is essential for *A. thaliana* plants effectively perform under Fe deficient conditions, and a reduction in lignin content was observed in seedlings with altered *miR408* expressions under Fe deficiency. Additionally, as all targets of miR408 are mRNAs that encode apoplastic cuproproteins, it was suggested that miR408 plays a role in Cu redistribution under Cu depletion [105,108]. However, miR408 mutant lines did not showed significant changes in SODs expression under Fe deficiency, showing that miR408 cannot act as regulator of metalloprotein substitution. Furthermore, plants OE-miR408 showed a reduced expression of *AtFIT* when cultivated under Fe deficiency, suggesting that for *AtFIT* expression, and the subsequent Fe deficiency responses, lower levels of *miR408* are required. On the other hand, knockdown mutants showed altered expression of *AtbHLH39*, which can also explain the defective Fe deficiency response [212].

Fe/Cu crosstalk also takes place in the mitochondria. Mitochondrial Cu chaperone 19, COX19, participates in the biogenesis of cytochrome c oxidase (COX) binding Cu ions in both yeast and humans, and may play a role in Cu insertion into COX [216,217]. Oxidative, biotic, and excess of Cu, Zn, and Fe stress induce *AtCOX19* [218]. However, Fe is required for alternative oxidase (AOX) function [219]. When COX pathway is limited, the importance of AOX respiration is emphasized [220]. Lines with decreased *AtCOX19* expression showed induced *AtCSD1* and repressed *miR398* expression under Cu deficiency, and showed significantly lower and higher Cu concentrations in roots and shoots, respectively, when compared with WT. *AtCOX19* also influences the Fe concentration of plants, as mutant lines showed higher shoot Fe concentration, compared with WT. Additionally, such lines showed compromised COX assembly and COX-dependent respiration under Fe deficiency [221].

Frataxin also seems to link Cu and Fe homeostasis [222]. Frataxin is a nuclear-encoded mitochondria/chloroplast-localized protein in *A. thaliana* [223–225]. In mitochondria, frataxin plays a role in Fe storage in Fe-S cluster biosynthesis, protection against oxidative stress, and ROS formation after Cu reduction and Fe metabolism, in the biosynthesis of heme. In chloroplasts frataxins are associated to Fe-S biosynthesis, with metal metabolism and Cu reduction, prevention of ROS accumulation, and response to Fe-S cluster oxidation (for a detailed review, see Gomez-Casati et al. 2018 [222]).

A. thaliana possesses two fumarase genes, *AtFUM1* and *AtFUM2*. *AtFUM1* encodes the mitochondrial isoform and participates in the tricarboxylic acid cycle, and *AtFUM2* shows higher fumarase activity in leaves and plays a role on the substantial fumarate accumulation during the day [226,227]. When *A. thaliana* is submitted to simultaneous

Fe and Cu deficiencies, the level of fumaric acid was significantly reduced [228]. *atfum2* knockout lines showed enhanced tolerance to simultaneous Fe and Cu deficiencies [228,229], higher chlorophyll content, and preserved photosynthetic performance under Fe or Fe/Cu deficiencies, compared to WT. Additionally, the symptoms observed when plants were submitted to both Fe and Cu deficiencies were not detected in *atfum2* plants, and WT and knockout lines possess the same concentration of Cu and Fe. The pool of Cu and Fe available for the photosynthetic machinery is increased in the *atfum2*. However, *atfum2* does not decrease FSD1 transcript level under simultaneous Cu/Fe deficiency, suggesting that perception of the Fe status is disturbed in the mutant lines under both deficiencies. This can be a result from a reduced levels of fumarate in *atfum2*, since fumarate plays a role on Fe an Mn chelation, and the reduced level of fumarate increases the amount of Fe available for incorporation into proteins in *atfuum2* [229]. These results add a new layer of information on the crosstalk between Fe and Cu and present new evidence of the response to both deficiencies.

5.2. Iron and Copper Crosstalk in Rice

Transcriptomic profiling of rice plants exposed to Cu excess showed that several genes involved in Fe homeostasis, such as *OsNAS1*, *OsNAS2*, *OsNAAT1*, and *OsIRO2*, are up-regulated. This suggests that Cu status crosstalks with Fe homeostasis as observed in *A. thaliana* [89,198]. Rice lines overexpressing *AtCOPT1* are more sensitive to Cu excess, show shoot and root growth inhibition, and higher expression of *OsHRZ1*, *OsHRZ2*, *OsIRO2*, and *OsFd1* in shoots. Moreover, OE-*AtCOPT1* lines were more sensitive to Fe and Cu deficiencies isolated or combined. Additionally, rice OE-*AtCOPT1* lines have decreases root Fe accumulation in both control and Cu excess conditions, suggesting that Fe deficiency signaling increases the Fe mobilization to sink organs, in agreement with the increased Fe concentration observed in polished and unpolished grains from plants overexpressing *AtCOPT1* [89]. These results reinforce the importance of Cu and Fe crosstalk and its possible application for biofortification.

6. Copper-Hyperaccumulating Plants

A hyperaccumulator plant can accumulate metals in their shoot dry matter, and the concentrations in shoots (10–500 times more than that in usual plants) are invariably greater than that in roots, showing a special ability of the plant to absorb and transport metals and store them in their above-ground part, specifically in the vacuoles of large epidermal cells [10,230–233]. Some of these hyperaccumulator plants could be used for the remediation of agricultural field polluted by heavy metals or mining sites [232,234]. Even though there are many reports of hyperaccumulating plants [235], few reports are related to Cu-hyperaccumulating ones, and true Cu hyperaccumulation in the sense of reaching thousands of ppm in the shoot has rarely been confirmed [2,232,234,236,237].

Considering the threshold of 1 $mg \cdot g^{-1}$ dry weight to meet the definition of Cu-hyperaccumulating plant [238], Jiang et al. (2004) [239] and Wang et al. (2004) [240] described the physiological responses of *Elsholtzia splendens* and *Commoelina communis*, respectively, to high Cu levels, with both species slightly exceeding the minimum limit to be considered a hyperaccumulator. *E. splendens* exhibited high tolerance to Cu toxicity in the soils, and normal growth was attained up to 1 $mg \cdot g^{-1}$ total soil Cu under glasshouse conditions. It was shown that Cu concentrations in the roots reached as high as 1.75 $mg \cdot g^{-1}$, which was 180 times greater than that in the shoots, limiting its commercial use for phytoextraction, but still useful for Cu phytostabilization or rhizofiltration in polluted areas [239]. On the other hand, *C. communis* accumulated more Cu (1.05 $mg \cdot g^{-1}$) on the leaves than on the roots after exposure for eight days under 1 mM Cu treatment, being able to tolerate such high Cu concentration for more than four months, with no symptoms of Cu toxicity [240].

Kobayashi et al. (2005) [241] showed that both the aboveground and underground parts of *Athyrium yokoscense* plants collected from an abandoned mine area contain high Cu concentrations (6.4 and 15 $mg \cdot g^{-1}$, respectively). Interestingly, in the aboveground

part, most of the Cu was found on the water-soluble material, while most of the Cu on the underground part was located at the residual lignin fraction. Such difference can be attributed to the cell wall of shoots and roots. Copper bound to shoot cell walls seems to be easily dissolved into the water-soluble fraction, while the root cell walls entrapped metals on the cell surfaces, bound to the high molecular weight lignin [241].

The amphibious plant *Crassula helmsii*, an aggressively invasive plant in Europe, accumulate more than 9 $mg \cdot g^{-1}$ in its shoots, clearly indicating that *C. helmsii* is an extremely efficient Cu-tolerant/hyperaccumulating plant [237]. Intriguingly, there seems to be no higher Cu requirement in *C. helmsii*, as previously detected for other metal-hyperaccumulating plants [242,243]. Chlorophyll to carotenoid conversion was not induced by Cu treatment, and their leaves bleached very quickly and showed an unusual rapid, and therefore, most likely active, degradation of all pigments. Such behavior was suggested to be a defense mechanism against Cu stress in *C. helmsii*, since sacrificing some Cu-filled leaves with recovery of nutrients from them could decrease Cu stress in the remaining tissues [237]. Additionally, *C. helmsii* seems to keep Cu out of the cytoplasm and sequester it into the vacuole, bound almost exclusively by oxygen ligands, probably organic acid malate originated by a shift from C3 to CAM photosynthesis metabolism [237].

Congo and Zambia hosts more than 30 known Cu hyperaccumulator plant species, which can accumulate extraordinarily high concentrations of Cu (and also cobalt (Co)) in their living tissues without showing any signs of toxicity. Van der Ent et al. (2019) [244] studied whether such abnormal Cu concentrations could be related to contamination of plant material with mineral particles. Using scanning electron microscopy with energy-dispersive spectroscopy (SEM-EDS) and herbarium specimens of *Haumaniastrum katangense*, *H. robertii*, and *Aeolanthus biformifolius*, it was found that these species are genuine Cu hyperaccumulators, being able to accumulate up to 1% of its weight in Cu. Interestingly, studies on *H. katangense* demonstrated that although it is extremely Cu-tolerant, it has excluder-type behavior under controlled conditions, despite Cu hyperaccumulation in field conditions [245]. Additionally, an enhanced requirement of Cu in the growth medium for optimum growth was also noted in these species. Therefore, we are not close to understanding the ecophysiology of this group of hyperaccumulators. It is important to highlight that no molecular mechanism of hyperaccumulation has been described yet in any of the putative Cu-hyperaccumulator plants [10].

7. Conclusions and Future Directions

With the projected increase in human population to at least 9 billion people by 2050, feeding everyone without increasing (and aiming to decrease) environmental impact will be mandatory. For that, we will need to growth plants adjusted to each climate, which includes precise nutrition. Although Cu is not a commonly deficient nutrient in farmlands, fine tuning of Cu homeostasis can improve production, and Cu toxicity derived from agronomical practices such as using Cu-based fungicides [70,71] will need to be revised. For that, we will need to further advance our understanding of Cu homeostasis in plants, and its interaction with other nutrients and with the environment.

Basic regulation of Cu uptake and distribution still has major gaps. We know little besides how primary uptake is performed, and this knowledge is only described in *A. thaliana* (Figure 2; [4]). Therefore, it is necessary to translate this to crops, determine how Cu uptake is performed, whether the mechanism is conserved, and how it is regulated. We also know little regarding Cu transport in aboveground tissues, which ultimately affect Cu delivery to animals (and humans) through the diet. Only a few genes were linked to Cu delivery to seed and reproductive organs [137,160], and the mechanism of Cu entry in the embryo is yet to be described. Moreover, Cu homeostasis in leaves is lagging compared to our understanding of it in roots. How Cu status is communicated by shoots to roots, which may be linked to light signaling [105], is also a promising avenue. In-depth studies such as those addressing the cell biology of Cu transport and homeostasis [125–127] should become

more common, as observed for other nutrients such as Fe, shedding light into how plants control Cu levels.

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