MINIREVIEW

Regulation of copper transport in *Arabidopsis thaliana*: a biochemical oscillator?

Lola Peñarrubia · Nuria Andrés-Colás ·
Joaquín Moreno · Sergi Puig

Abstract Plants are among the most versatile higher eukaryotes in accommodating environmental copper availability to largely variable demands. In particular, copper deficiency in soils is a threat for plant survival since it mostly affects reproductive structures. One of the strategies that plant cells use to overcome this situation is to increase copper levels by expressing high-affinity copper transporters delivering the metal to the cytosol. In this minireview, we discuss recent advances in the structure, function, and regulation of the CTR/COPT family of copper transporters, and pay special attention to the *Arabidopsis thaliana* counterparts. These are constituted by transmembrane polypeptides, containing several copper-binding sequences of functional and/or regulatory value, and assembling as trimers. Copper deficiency activates the expression of some members of the COPT family via the interaction of the SPL7 transcription factor with reiterative GTAC motifs present in their promoters. Interestingly, the regulation of the synthesis of these transporters by copper itself constitutes a negative-feedback loop that could cause a sustained oscillation in the cytosolic copper levels. We analyze the theoretical conditions required for this hypothetical copper oscillation and the potential advantages of synchronization with other cycles. Diverse data in other organisms point to the relationship between copper homeostasis and circadian cycles.

Keywords *Arabidopsis* · Copper transport · Homeostasis · Biochemical oscillator · Circadian rhythms

This article will be printed in the upcoming Journal of Biological Inorganic Chemistry special issue Cell Biology of Copper.

L. Peñarrubia (✉) · N. Andrés-Colás · J. Moreno · S. Puig
Departament de Bioquímica i Biologia Molecular,
Universitat de València,
Avda. Dr. Moliner, 50,
46100 Burjassot, Valencia, Spain
e-mail: lola.penarrubia@uv.es; penarrub@uv.es

Introduction

Plants use copper (Cu) as a redox cofactor for a wide range of physiological processes, including photosynthesis, mitochondrial respiration, superoxide scavenging, cell wall metabolism, and ethylene perception. Besides being essential, Cu is a potential toxic agent when in excess. The reactivity of Cu ions can lead to the generation of harmful reactive oxygen species via Fenton and Haber–Weiss reactions that cause severe oxidative damage to cells [1]. Cu is also at the top of the Irving–Williams series and tightly binds to target sites in polypeptides [2]. Owing to their sessile nature, plants are probably among the best adapted higher eukaryotes to accommodate their Cu requirements to the wide range of environmental Cu availability. The necessity for Cu varies not only during the daily cycle as photosynthesis is probably the main metal-requiring process, but also throughout plant development green tissues and reproductive organs become strong Cu nutrient sinks. To conciliate the dynamic changes in Cu supply and demands, plants have evolved sophisticated and tightly regulated homeostatic networks that control Cu uptake, delivery to target proteins, and detoxification. In *Arabidopsis* cells, Cu^+ transport toward the cytosol is mediated by the high-affinity COPT1–COPT6 transporter family, and Cu^+ efflux occurs through P-type ATPases, including RAN1, PAA1, PAA2, HMA5,

and HMA1. Metallochaperones, including ATX1, CCH, and CCS1, mediate Cu^+ delivery to specific protein targets (reviewed in [3–5]). Although Cu homeostasis network components, primarily described in the yeast *Saccharomyces cerevisiae,* are widely conserved among eukaryotes, the transcription factors involved in Cu responses are divergent. Because plants constitute one of the main entrances of micronutrients into trophic chains, deciphering the regulatory mechanisms underlying Cu uptake and distribution to edible products is relevant, to avoid toxic Cu levels in agronomic plants when grown on Cu-contaminated soils [6]. The Cu levels in shoots considered adequate are 6 µg g^{-1} dry weight, and correspond to 6.2 µM in fresh tissue [7]. This global concentration of endogenous Cu is relatively high and does not reflect a homogeneous distribution. Once mature cells have attained a sufficient Cu level, the transporters involved in Cu efflux probably drive the export of excess Cu to the vacuole and the cell wall, which become the main Cu storage sites in cultured cells [8]. Endogenous Cu storage is not only important for detoxification, but is also a repository source to allow further growth during eventual Cu-deficiency stages. Although plants act as effective miners when Cu is scarce, under

severe restrictions they can show diverse deficiency symptoms, which mostly affect fruit production and cause important economic losses in agriculture [7].

Copper transport by the *Arabidopsis* COPT family

In response to Cu deficiency, eukaryotes activate different mechanisms to increase the availability of this essential cofactor. One such mechanism is the expression of a conserved family of small integral membrane proteins, known as the CTR family of Cu transporters, which mediate high-affinity Cu transport into the cytosol of eukaryotic cells (reviewed in [9, 10]). Functional complementation studies in yeast strains defective for high-affinity Cu uptake (*ctr1Δ* strains) have identified CTR proteins in other organisms. The model plant *Arabidopsis thaliana* contains a family of six CTR proteins, known as the COPT family of *cop*per *t*ransporters (Fig. 1, COPT1–COPT6) [6, 11]. COPT1 was the first COPT family member to be identified and characterized [12, 13]. Several lines of evidence indicate that COPT1 functions in soil Cu uptake in *Arabidopsis* plants when Cu becomes limited [12]. Firstly, COPT1 is
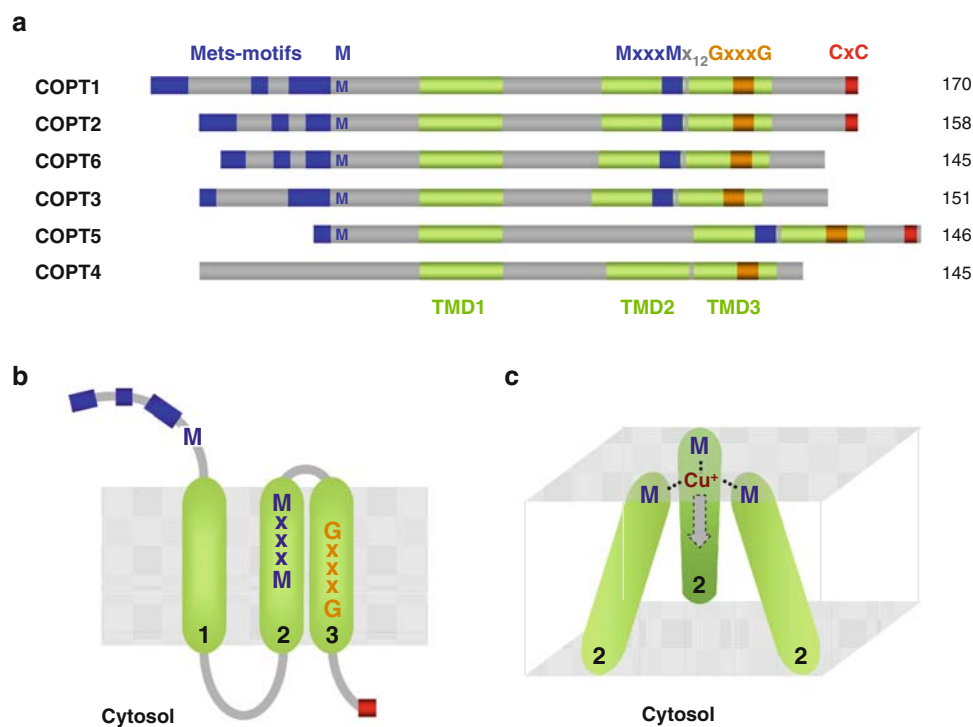

Fig. 1 The *Arabidopsis* COPT family of Cu transport proteins. **a** Alignment of the putative *Arabidopsis thaliana* CTR-type Cu transporters. The most relevant characteristics in COPT proteins are represented from the amino terminus (*left*) to the carboxy terminus (*right*). The conserved features include three transmembrane domains (*TMD1*, *TMD2*, *TMD3*; in *green*), methionine-rich motifs (*Mets-motifs*; consisting of methionine and histidine residues separated by

two or fewer amino acids) and MxxxM motifs (in *blue*), GxxxG motifs (in *orange*), and CxC cysteine motifs (in *red*). The predicted amino acid length is shown on the *right*. **b** The proposed topological structure of the CTR/COPT proteins. **c** The spatial disposition of TMD2 in the human Ctr1 homotrimer complex. Crucial methionines from different monomers could coordinate Cu^+ during its transport toward the cytosol

expressed in both primary and secondary root tips. Secondly, *COPT1* messenger RNA levels increase when Cu availability diminishes. Thirdly, *COPT1*-defective plants present a 50% decrease in Cu acquisition and accumulation, as well as significant growth defects on Cu-deficient media. Furthermore, the elevated expression levels of *COPT1* messenger RNA in pollen grains and the pollen defects observed in *COPT1*-defective plants indicate a crucial function of Cu and COPT1 in pollen development [12]. Multiple data accumulated over the past decade indicate that CTR proteins are highly specific for reduced Cu^+. Evidence includes the requirement of membrane-associated metalloreductases, the enhancement of CTR-associated Cu transport upon addition of reductants, and the efficient inhibition of Cu uptake by competition with the isoelectric Ag^+ cation, but not with divalent metal ions [14, 15]. This seems to be the case for COPT1, whose ^{64}Cu uptake rate in yeast is significantly inhibited when excess cold Cu or Ag is added [11]. Although the identity of the cell-surface Cu-reduction system in plants is currently unknown, potential candidates include the *Arabidopsis* family of FRO metalloreductases with a demonstrated function in iron assimilation [16, 17]. Interestingly, expression of the *Arabidopsis FRO3* gene is induced upon Cu limitation, whereas *FRO6* levels are reduced [16].

Topological in vivo studies have demonstrated that CTR proteins contain three transmembrane domains (TMDs) with the amino terminus and the carboxy terminus localized toward the extracellular space and the cytosol, respectively [18–20] (see Fig. 1b for COPT topology). The genetic and biochemical evidence from yeast CTRs suggests that the function of extracellular methionine-rich motifs (Mets-motifs in Fig. 1) is to selectively sequester Cu^+ ions from an oxidizing environment and stabilize them by coordinating the metal to thioester groups in methionine residues for their subsequent translocation to the cytosol [18, 21, 22]. All *Arabidopsis* COPT family members, with the exception of COPT4, contain putative methionine-rich motifs within their amino-terminal region (Fig. 1a). It should be noted that the methionine-rich motifs in *Arabidopsis*, humans, and flies, but not yeast, are also rich in histidine residues. Furthermore, two recently identified CTR transporters from the unicellular green alga *Chlamydomonas reinhardtii*, CrCTR1 and CrCTR2, possess six putative Cu-binding motifs, which contain both methionine and cysteine residues that would increase the scavenging capacity of the transporter [23].

Essential residues for Cu transport include a methionine allocated at approximately 20 amino acids before TMD1 (methionine 127 in yeast Ctr1; yCtr1) and an MxxxM motif within TMD2. The mutagenesis of these methionines to alanine or leucine, but not to serine or histidine, abrogates most Cu transport capacity in yeast and human CTR proteins, suggesting that Cu transiently coordinates to these residues during its translocation pathway to the cytosol [18]. Although these studies have not been performed in *Arabidopsis*, all the COPT family members that fully or partially complement the respiratory defect of a yeast *ctr1Δ* strain contain these critical methionines (see Fig. 1 for motifs in COPTs). The only *Arabidopsis* COPT protein that does not contain any of these essential methionines and that therefore does not complement yeast *ctr1Δ* cells [11] is COPT4, which otherwise exhibits a fairly conserved amino acid sequence including three putative TMDs (Fig. 1).

The biochemical and genetic evidence collected over the past 10 years by different laboratories suggests that human and yeast CTR proteins oligomerize and assemble at the membrane as trimers [18, 20, 24–26]. The mutagenesis in yCtr1 of a strictly conserved GxxxG motif within TMD3 indicates that this motif acts as a glycine zipper, which is essential for close helix packing and, therefore, for trimer assembly and delivery of the functional transporter to its final destination [27]. It is tempting to speculate that COPT4, which contains the GxxxG motif but no crucial methionines, could perform a regulatory function by interacting with other COPT proteins by inhibiting their Cu-transporting capacity. Recent structural data with the human Ctr1 (hCtr1) protein show that it assembles as symmetrical homotrimers with a cone-shaped pore in the center [28]. The second TMD, and especially its MxxxM motif, is crucial to define the narrowest end (approximately 8 Å) of the pore toward the extracellular side of the membrane (Fig. 1c). The mutagenesis of a methionine residue within the MxxxM motif to cysteine enables the formation of disulfide bonds between cysteines from different monomers, which highlights the close proximity between the MxxxM motifs in the structure [28]. In addition, genetic and biochemical data also suggest that Cu^+ may simultaneously coordinate to three methionines from different hCtr1 monomers during the passage of the metal through the pore [28]. Genetic evidence from yCtr1 also indicates cooperation between monomers during the Cu transport mechanism. As the yeast cells which express a Ctr1 protein mutagenized at the extracellular methionine residue 127 or at the MxxxM motif within TMD2 are unable to transport Cu, the cells coexpressing both mutants display a significant Cu uptake capacity [18]. Although many of the structural and functional characteristics described for yCtr1 and hCtr1 could function in other CTR proteins, including the *Arabidopsis* COPT family members, it should be noted that the conservation of the primary amino acid sequence between the CTR proteins is restricted to the $MxxxMx_{12}GxxxG$ signature motif (Fig. 1). Therefore, it is possible that each transporter has developed slightly different solutions to accommodate the requirements for assembly and Cu transport.

Recent advances are contributing to clarification of the role of the intracellular face of CTR proteins in their regulation and function. The structural data obtained for hCtr1 show that the contact between the subunits on the cytosolic side is established by interactions between TMD2 and TMD3 from adjacent monomers [28]. The findings of studies with yCtr1 and hCtr1 indicate that although the cytosolic carboxy-terminal domain (CTD) of these transporters is dispensable for Cu transport, it is important to protect cells against toxic concentrations of Cu in the environment [29, 30]. In this sense, yeast cells expressing a Ctr1 protein with a CTD deletion are hypersensitive to Cu [30]. The function of the CTD in Cu excess could merely be to mediate Ctr1 endocytosis and degradation as has been described [29, 31–33]. However, recent complementation and ^{64}Cu uptake experiments in yeast demonstrate that the CTD mediates a rapid block of yCtr1 Cu transport in response to excess Cu [30]. It has been postulated that cysteine-rich motifs within the yCtr1 CTD, which bind Cu^+ in vitro [34] and confer Cu resistance when overexpressed [30], could sense elevated intracellular Cu levels and, therefore, inactivate Ctr1-mediated Cu transport. Interestingly, many CTR proteins, including *Arabidopsis* COPT1, COPT2, and COPT5, contain cysteine residues within their CTD (CxC motif in Fig. 1). Other than the regulatory function, the CTD could also participate in the mechanism of Cu^+ transfer to conserved cytosolic metallochaperones, such as ATX1 and CCS1, which are responsible for Cu delivery to the secretory pathway and the Cu/zinc superoxide dismutase (Cu/ZnSOD), respectively. In vitro studies demonstrated Cu exchange between the CTD of yCtr1 and yeast Atx1 metallochaperone, suggesting a direct Cu^+ transfer between these two proteins [35].

Current data are consistent with a model for CTR-mediated Cu transport in which Cu^+ is firstly recruited to the protein by extracellular methionine-rich motifs, secondly it is transported through the central pore by Cu-exchange reactions between defined Cu^+-binding sites including the MxxxM motif in TMD2 and cysteine residues in the CTD, and thirdly it is transferred to cysteines within cytosolic metallochaperones. Evidence from trypsin digestions of hCtr1 and fluorescence resonance transfer studies with yCtr1 suggests that the passage of Cu through the protein and its transfer to cytosolic components is associated with well-defined conformational changes [19, 36]. Since, biochemical evidence obtained with hCtr1 indicates that CTR-mediated Cu transport is ATP-independent [15], the driving force for Cu import could be the higher stability of intracellular Cu^+–cysteine interactions compared with extracellular Cu^+–methionine interactions, as well as the elevated Cu-chelating capacity of the cytosol.

Other than the plasma-membrane-associated CTR proteins that participate in cellular Cu acquisition, *S. pombe* and *S. cerevisiae* possess intracellular CTR proteins that localize to the membrane of the vacuole and mediate Cu mobilization in response to extreme Cu limitation [37, 38]. Similarly to plasma membrane CTR proteins, the vacuolar *S. cerevisiae* Ctr2 protein requires the participation of a Cu metalloreductase to perform its Cu transport function [39]. The forced plasma membrane localization of the vacuolar yeast Ctr2 protein induced by random mutagenesis further supports that its mechanism of Cu transport is similar to that of cell-surface transporters [38]. To date, however, the signals that mediate the delivery of CTR proteins to the vacuole or to another intracellular location have not yet been identified, thus making any in silico prediction of CTR localization difficult. The subcellular localization of *Arabidopsis* COPT proteins has not yet been reported. However, the functional complementation of *ctr1Δ* yeast mutants suggests that COPT1 and COPT2, which fully complement *ctr1Δ* cells, could localize to the plasma membrane, whereas COPT3 and COPT5, which only partially complement the respiratory defect of yeast *ctr1Δ*, are probably intracellularly localized [11]. In many unicellular organisms, including *S. cerevisiae*, *S. pombe*, and *C. reinhardtii*, as well as multicellular organisms such as *Drosophila melanogaster* and *A. thaliana*, plasma membrane CTR genes are highly upregulated in response to Cu deficiency (reviewed in [4, 6, 10, 40, 41]). Although the mechanisms that mediate the CTR/COPT transcriptional control in different organisms may be quite diverse, in the next section we review recent advances in the characterization of mechanisms that mediate the Cu regulation of *COPT1*, *COPT2*, and of other *Arabidopsis* genes.

Regulation of copper homeostasis in response to copper deficiency

When Cu is limited, one of the strategies aimed to save Cu for essential cuproproteins is the substitution of the dispensable ones for other metalloproteins, which, by using a different metal (usually iron), perform a similar task. A well-known example is the replacement of chloroplastic Cu/ZnSOD with the iron counterpart, iron superoxide dismutase (FeSOD), to probably allocate Cu preferentially to the indispensable plastocyanin [42]. This metalloenzyme substitution is considered a hallmark strategy that underlines Cu deficiency responses in *Arabidopsis*, as well as in other plants [43].

The increase in the expression of FeSOD and of several other genes involved in the metal homeostasis in response to low Cu levels has been attributed to the presence of reiterative *cis* regulatory elements (GTAC motifs) present

in their promoters (Fig. 2) [44–46]. The *trans* factors involved in the transcriptional activation in Cu deficiency belong to the *SQUAMOSA* promoter binding protein (SBP) family. SBPs were originally identified as the factors which bind the promoter of the MADS-box floral meristem identity gene *SQUAMOSA* in *Antirrhinum majus* [47]. SBPs form a diverse family of transcription factors specific to photosynthetic eukaryotes, ranging from *Chlamydomonas* to higher plants [44–46]. The SBP domain contains two particular types of zinc-finger binding sites formed by eight cysteine and histidine residues. Except for the SBP domain, the rest of the protein is quite divergent within the different family members [48]. *Arabidopsis* has a 17-member *SQUAMOSA* promoter binding like (SPL) protein family of transcription factors [48]. Among the diverse *Arabidopsis* SPL proteins, the SBP domain in SPL7 shows the highest similarity to the SBP domain in the Cu response regulator 1 (Crr1) transcription factor which regulates Cu deficiency responses in *Chlamydomonas* [44]. Recent data on *Arabidopsis* indicate that the transcriptional activation of the different target genes involved in the response to Cu deficiency, including those coding for the FeSOD and the high-affinity Cu transporters COPT1 and COPT2, is mediated by SPL7 through the interaction with the reiterative GTAC motifs present in their promoters [46]. As a result,

cytosolic Cu uptake through the COPT transporters is transcriptionally activated when there is a low environmental Cu supply (Fig. 2).

As additional targets of the same regulatory response to Cu deficiency, there is also a group of microRNAs that target transcripts for cuproteins, such as the Cu/ZnSOD, plantacyanin, and laccases (Fig. 2). These are collectively known as Cu-microRNAs and include miR397, miR398, miR408, and miR857 [46, 49–52]. These Cu-microRNAs present the reiterative GTAC motifs in their promoters that allow their transcriptional activation in Cu deficiency by SPL7 interaction. Cuproteins that are targeted by Cu-microRNAs are considered nonessential and are reciprocally regulated by Cu in contrast to the microRNAs. This means that they accumulate only if the Cu supply is sufficient to fulfill the demands of essential cuproteins [50, 52]. This strategy contributes to the organization of a dynamic and hierarchic use of Cu in cuproteins in accordance with their essentiality, the environmental Cu availability, and the physiological plant demands. Despite the expression of microRNAs apparently being a widely used strategy in higher plants under nutrient deficiency, the target proteins are different. Thus, with sulfur and phosphorus nutrient deficiencies, microRNA targets are part of the machinery responsible for assimilation. The fact that
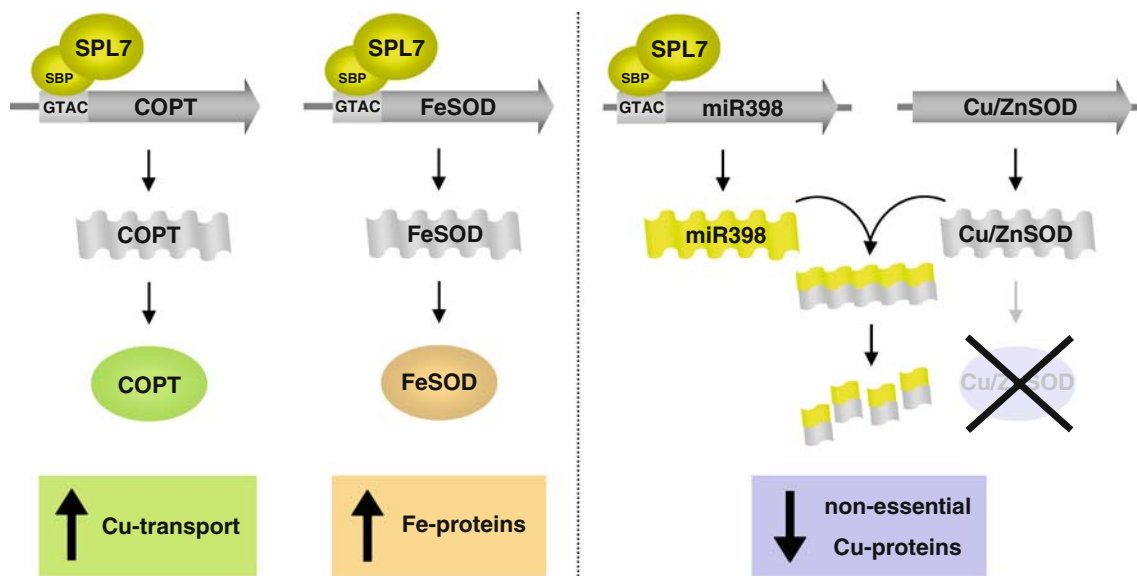

Fig. 2 The response to Cu deficiency in *A. thaliana*. *SQUAMOSA* promoter binding like protein 7 *(SPL7)* mediates the transcriptional activation of the genes involved in Cu homeostasis, through binding of its *SQUAMOSA* promoter binding protein *(SBP)* domain to the GTAC motifs present in the promoters of the target genes. These targets include the high-affinity Cu uptake transporters *COPT1* and *COPT2* *(COPT)*. As a result, *Arabidopsis* plants respond to Cu deficiency by increasing their Cu uptake capacity. The iron superoxide dismutase *(FeSOD)* gene is another SPL7 target that is upregulated in low Cu conditions, probably to substitute its nonessential cuproprotein counterpart (Cu/Zn superoxide dismutase, *Cu/ZnSOD*). In response to low Cu supply, the SPL7 transcription factor also activates the expression of microRNAs: miR397, miR398, miR408, and miR857 (Cu-microRNAs). Then, Cu-microRNAs drive the degradation of messenger RNAs encoding nonessential cuproteins. As an example, miR398 and the degradation of its target, the Cu/ZnSOD, is shown. As a consequence, the synthesis of nonessential Cu proteins decreases to obtain a better balance between nutritional supply and physiological demands

only Cu-microRNAs mediate the downregulatory effects on cuproteins has been attributed to the tight Cu binding capacity of the target proteins, which forces their elimination when Cu becomes scarce (reviewed in [3]).

Changes in gene expression under Cu deficiency in both *Arabidopsis* and *Chlamydomonas* have been attributed to the function of SBPs as transcriptional activators. Sufficient Cu has been suggested to possibly cause a change in SBP binding and/or activity by either a displacement of zinc by Cu at the zinc-finger domain or Cu binding to a metallothionein-like domain present at the carboxy-terminal part of the Crr1 protein [44]. However, the regulation of Cu homeostasis factors by Cu in bryophytes has been attributed to the negative regulation (repression under high Cu conditions) of the *Physcomitrella patens* transcription factor *Pp*SBP2 [45]. Therefore, although recent progress made in transcriptional regulation of Cu-microRNAs and SPL7 functioning has notably contributed to our understanding of the responses under Cu deficiency, the picture is far from complete. Both the specific requirements in the *cis* elements and the diverse functions of the *trans*-acting factors deserve further study to understand how their combinatorial effects contribute to the adaptive adjustment to various Cu environmental conditions.

Copper autoregulatory feedback loop

In a highly simplified model, the cytosolic Cu concentration depends on the balance between influx transport (mostly due to COPT family members under a low Cu supply) and efflux transport, either outside the cell or to storage compartments (driven by pumps). As mentioned previously, the expression of several members of the *Arabidopsis* COPT family, including *COPT1* and *COPT2*, is downregulated by Cu [11]. Repeated GTAC motifs within their promoters could account for the SPL-mediated expression under Cu deficiency [46]. Remarkably, the COPT-mediated Cu transport activity and the Cu-inhibited expression of COPT genes integrate a negative autoregulatory feedback loop. This loop could serve a homeostatic function (i.e., to stabilize a steady concentration of cytosolic Cu), but it could also fulfill the conditions of a biochemical oscillator [53] if the negative-feedback signal is sufficiently delayed (through COPT transcription and translation, and Cu transport) to avoid settling on a stable steady state. A more detailed analysis of this possibility is given in the electronic supplementary material. Briefly, a low cytosolic Cu level induces the synthesis of COPT transporters (Fig. 3a), releasing Cu from internal stores. Once a high cytosolic level of Cu has been achieved, the synthesis of COPTs is inhibited (Fig. 3a), and their turnover allows the pumping of the Cu back to storage sites,
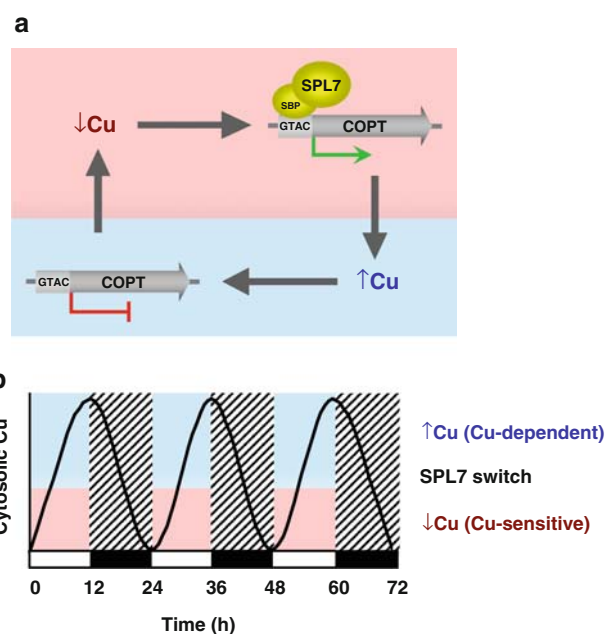
Fig. 3 Proposed model for the oscillation of cytosolic Cu concentration and its interaction with circadian rhythms. **a** Model for the transcriptional regulation of the COPT transporters and the resulting cytosolic Cu oscillation. SPL7 functions under low Cu supply as a transcriptional activator of the *COPT1* and *COPT2* (*COPT*) transporters through binding to the GTAC motifs within their promoters. As a consequence of COPT expression, Cu uptake to the cytosol would increase until high Cu levels prevent SPL7 function. Afterwards, COPT turnover without replacement would decrease the cytosolic Cu influx, allowing the efflux pumps to reinstate the initial low Cu conditions. **b** Oscillation in the cytosolic Cu levels and its synchronization to the circadian rhythm. The Cu feedback loop shown in **a** might cause a cytosolic biochemical Cu oscillation around the concentration responsible for the sensor SPL7 transcriptional switch. The period of this oscillation may be synchronized to the environmental or endogenous rhythms represented here as the light/dark cycle (see the text for further details)

returning the Cu level to the initial low level in the cytosol. Taking reasonable assumptions for turnover rates, efflux pumping, and Cu concentrations at both compartments, the system produces a cytosolic Cu oscillation, based on a delayed negative-feedback loop. The theoretical requirements and characteristics of this self-sustained oscillation of cytosolic Cu levels have been mathematically analyzed (see the electronic supplementary material for details). The oscillation occurs for a certain range of values of the parameters which, while plausible, still await to be experimentally ascertained. Unfortunately, a complex Cu compartmentalization pattern and the lack of a reliable endogenous indicator of the cytosolic Cu level currently hinder any straightforward experimental determination of the presumed oscillation. If Cu levels oscillate, a still unanswered question is the exact location of the Cu store involved in the oscillatory mechanism. In this sense, vacuoles or internal endocytic pathway derived vesicles are the

main candidates to serve this task, where the efflux transporters would pump Cu and members of the COPT family could participate in the return Cu influx. It is likely that even plasma membrane COPT transporters could be internalized to intracellular vesicles in plants, as previously described for CTRs in other organisms [29, 32, 33]. In this sense, the subcellular localization of the different COPT family members is crucial to assess their role in this process.

Although the cytosolic Cu oscillation can be of small amplitude, it must encompass the critical concentration triggering the sensor SPL7 regulatory switch (Fig. 3b), which is part of the feedback mechanism. As a consequence, transcription of additional target genes would operate cyclically. This would allow the scheduling of favorable processes taking place at the highest Cu concentrations (Cu-dependent) in preference to those processes which are more prone to occur at the lowest Cu levels (Cu-sensitive) at different phases of the cycle (Fig. 3b). An additional advantage would result from the synchronization of the putative Cu oscillation to other cellular cycles, since living beings obtain adaptive advantages when their metabolic functions are arranged in a coherent schedule tuned to environmental rhythms [54]. The presence of a GTAC motif, which has been shown to be sufficient for the Cu-deficiency response [46], within the promoters of the nuclear components of the *Arabidopsis* central oscillator suggests a plausible explanation as to how Cu affects the expression of the circadian clock. In turn, the *COPT2* and *COPT1* promoters also possess putative circadian elements, suggesting an input from the clock in the oscillation of the expression of the high-affinity Cu transporters. If these putative regulatory elements are functional at the transcriptional level, the reciprocal regulation between Cu transport and the circadian clock could lead to synchronization and inseparable outputs from the two coupled cycles.

Finally, several reports reinforce the interconnection between Cu and the circadian rhythms in organisms other than *Arabidopsis*. Two members of the *P. patens* SPL family are regulated by the circadian clock [55]. In *Neurospora crassa*, a Cu metallothionein expression follows a circadian cycle [56]. In mammals, the expression of a pineal ATPase, a variant of a Cu transporter, also displays a dramatic circadian rhythm [57]. Metal-binding prion proteins are involved in Cu metabolism and their absence has been associated with alterations in sleep and the daily rhythms of many hormones [58]. Should this mutual influence between Cu homeostasis and the circadian rhythms be an extended feature among higher eukaryotes, as the above observations suggest, a wealth of new experimental approaches could be envisaged to further address important open questions, such as the role of Cu in

neurodegenerative disorders in humans, by introducing the temporal dimension in Cu-dependent processes of the cell.

References

1. Halliwell B, Gutteridge JM (1984) Biochem J 219:1–14
2. Lippard SJ, Berg JM (1994) Principles of bioinorganic chemistry. University Science Books, Mill Valley
3. Burkhead JL, Reynolds KA, Abdel-Ghany SE, Cohu CM, Pilon M (2009) New Phytol 182:799–816
4. Pilon M, Cohu CM, Ravet K, Abdel-Ghany SE, Gaymard F (2009) Curr Opin Plant Biol 12:347–357
5. Puig S, Penarrubia L (2009) Curr Opin Plant Biol 12:299–306
6. Puig S, Andres-Colas N, Garcia-Molina A, Penarrubia L (2007) Plant Cell Environ 30:271–290
7. Märschner H (2002) Mineral nutrition in higher plants. Academic Press, London
8. Bernal M, Ramiro MV, Cases R, Picorel R, Yruela I (2006) Physiol Plant 127:312–325
9. Puig S, Thiele DJ (2002) Curr Opin Chem Biol 6:171–180
10. Kim BE, Nevitt T, Thiele DJ (2008) Nat Chem Biol 4:176–185
11. Sancenon V, Puig S, Mira H, Thiele DJ, Penarrubia L (2003) Plant Mol Biol 51:577–587
12. Sancenon V, Puig S, Mateu-Andres I, Dorcey E, Thiele DJ, Penarrubia L (2004) J Biol Chem 279:15348–15355
13. Kampfenkel K, Kushnir S, Babiychuk E, Inze D, Van Montagu M (1995) J Biol Chem 270:28479–28486
14. Hassett R, Kosman DJ (1995) J Biol Chem 270:128–134
15. Lee J, Pena MM, Nose Y, Thiele DJ (2002) J Biol Chem 277:4380–4387
16. Mukherjee I, Campbell NH, Ash JS, Connolly EL (2006) Planta 223:1178–1190
17. Wu H, Li L, Du J, Yuan Y, Cheng X, Ling HQ (2005) Plant Cell Physiol 46:1505–1514
18. Puig S, Lee J, Lau M, Thiele DJ (2002) J Biol Chem 277:26021–26030
19. Eisses JF, Kaplan JH (2002) J Biol Chem 277:29162–29171
20. Klomp AE, Juijn JA, van der Gun LT, van den Berg IE, Berger R, Klomp LW (2003) Biochem J 370:881–889
21. Beaudoin J, Laliberte J, Labbe S (2006) Microbiology 152:209–222
22. Jiang J, Nadas IA, Kim MA, Franz KJ (2005) Inorg Chem 44:9787–9794
23. Page MD, Kropat J, Hamel PP, Merchant SS (2009) Plant Cell 21:928–943
24. Dancis A, Haile D, Yuan DS, Klausner RD (1994) J Biol Chem 269:25660–25667
25. Pena MM, Puig S, Thiele DJ (2000) J Biol Chem 275:33244–33251
26. Aller SG, Unger VM (2006) Proc Natl Acad Sci USA 103:3627–3632
27. Aller SG, Eng ET, De Feo CJ, Unger VM (2004) J Biol Chem 279:53435–53441
28. De Feo CJ, Aller SG, Siluvai GS, Blackburn NJ, Unger VM (2009) Proc Natl Acad Sci USA 106:4237–4242

29. Liu J, Sitaram A, Burd CG (2007) Traffic 8:1375–1384
30. Wu X, Sinani D, Kim H, Lee J (2009) J Biol Chem 284:4112–4122
31. Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD (1996) EMBO J 15:3515–3523
32. Petris MJ, Smith K, Lee J, Thiele DJ (2003) J Biol Chem 278:9639–9646
33. Guo Y, Smith K, Lee J, Thiele DJ, Petris MJ (2004) J Biol Chem 279:17428–17433
34. Xiao Z, Loughlin F, George GN, Howlett GJ, Wedd AG (2004) J Am Chem Soc 126:3081–3090
35. Xiao Z, Wedd AG (2002) Chem Commun 588–589
36. Sinani D, Adle DJ, Kim H, Lee J (2007) J Biol Chem 282:26775–26785
37. Bellemare DR, Shaner L, Morano KA, Beaudoin J, Langlois R, Labbe S (2002) J Biol Chem 277:46676–46686
38. Rees EM, Lee J, Thiele DJ (2004) J Biol Chem 279:54221–54229
39. Rees EM, Thiele DJ (2007) J Biol Chem 282:21629–21638
40. Balamurugan AN, Chang Y, Bertera S, Sands A, Shankar V, Trucco M, Bottino R (2006) Diabetologia 49:1845–1854
41. Merchant SS, Allen MD, Kropat J, Moseley JL, Long JC, Tottey S, Terauchi AM (2006) Biochim Biophys Acta 1763:578–594
42. Abdel-Ghany SE, Muller-Moule P, Niyogi KK, Pilon M, Shikanai T (2005) Plant Cell 17:1233–1251
43. Cohu CM, Pilon M (2007) Physiol Plant 129:747–755
44. Kropat J, Tottey S, Birkenbihl RP, Depege N, Huijser P, Merchant S (2005) Proc Natl Acad Sci USA 102:18730–18735
45. Nagae M, Nakata M, Takahashi Y (2008) Plant Physiol 146:1687–1696
46. Yamasaki H, Hayashi M, Fukazawa M, Kobayashi Y, Shikanai T (2009) Plant Cell 21:347–361
47. Klein J, Saedler H, Huijser P (1996) Mol Gen Genet 250:7–16
48. Birkenbihl RP, Jach G, Saedler H, Huijser P (2005) J Mol Biol 352:585–596
49. Sunkar R, Kapoor A, Zhu JK (2006) Plant Cell 18:2051–2065
50. Yamasaki H, Abdel-Ghany SE, Cohu CM, Kobayashi Y, Shikanai T, Pilon M (2007) J Biol Chem 282:16369–16378
51. Dugas DV, Bartel B (2008) Plant Mol Biol 67:403–417
52. Abdel-Ghany SE, Pilon M (2008) J Biol Chem 283:15932–15945
53. Novak B, Tyson JJ (2008) Nat Rev Mol Cell Biol 9:981–991
54. Dodd AN, Gardner MJ, Hotta CT, Hubbard KE, Dalchau N, Love J, Assie JM, Robertson FC, Jakobsen MK, Goncalves J, Sanders D, Webb AA (2007) Science 318:1789–1792
55. Riese M, Zobell O, Saedler H, Huijser P (2008) Planta 227:505–515
56. Bell-Pedersen D, Shinohara ML, Loros JJ, Dunlap JC (1996) Proc Natl Acad Sci USA 93:13096–13101
57. Borjigin J, Payne AS, Deng J, Li X, Wang MM, Ovodenko B, Gitlin JD, Snyder SH (1999) J Neurosci 19:1018–1026
58. Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, Moser M, Oesch B, McBride PA, Manson JC (1996) Nature 380:639–642