Deregulated Copper Transport Affects Arabidopsis Development Especially in the Absence of Environmental Cycles\textsuperscript{1[C][W]}

Nuria Andrés-Colá\textsuperscript{2}, Ana Perea-García\textsuperscript{2}, Sergi Puig, and Lola Peñarrubia\textsuperscript{*}

Departament de Bioquímica i Biologia Molecular, Universitat de València, E–46100 Burjassot, Valencia, Spain

Copper is an essential cofactor for key processes in plants, but it exerts harmful effects when in excess. Previous work has shown that the Arabidopsis (Arabidopsis thaliana) COPT1 high-affinity copper transport protein participates in copper uptake through plant root tips. Here, we show that COPT1 protein localizes to the plasma membrane of Arabidopsis cells and the phenotypic effects of transgenic plants overexpressing either COPT1 or COPT3, the latter being another high-affinity copper transport protein family member. Both transgenic lines exhibit increased endogenous copper levels and are sensitive to the copper in the growth medium. Additional phenotypes include decreased hypocotyl growth in red light and differentially affected flowering times depending on the photoperiod. Furthermore, in the absence of environmental cycles, such as light and temperature, the survival of plants overexpressing COPT1 or COPT3 is compromised. Consistent with altered circadian rhythms, the expression of the nuclear circadian clock genes CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYCOTYL (LHY) is substantially reduced in either COPT1- or COPT3-overexpressing plants. Copper affects the amplitude and the phase, but not the period, of the CCA1 and LHY oscillations in wild-type plants. Copper also drives a reduction in the expression of circadian clock output genes. These results reveal that the spatiotemporal control of copper transport is a key aspect of metal homeostasis that is required for Arabidopsis fitness, especially in the absence of environmental cues.

\textsuperscript{1} This work was supported by the Spanish Ministry of Science and Innovation (grant nos. BIO2008–02835 and CSD2007–00057 to L.P. and predoctoral Formació del Personal Investigador fellowship to A.P.-G.), by Fondo Europeo de Desarrollo Regional funds and Innovation (grant nos. BIO2008–02835 and CSD2007–00057 to S.P.); and by the Fundació General de la Universitat de València (to S.P.).

\textsuperscript{2} These authors contributed equally to the article.

* Corresponding author; e-mail penarrub@uv.es.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Lola Peñarrubia (penarrub@uv.es).

\textsuperscript{[C]} Some figures in this article are displayed in color online but in black and white in the print edition.

\textsuperscript{[W]} The online version of this article contains Web-only data. www.plantphysiol.org/cgi/doi/10.1104/pp.110.153676

\textsuperscript{170} Plant Physiology\textsuperscript{®}, May 2010, Vol. 153, pp. 170–184, www.plantphysiol.org © 2010 American Society of Plant Biologists Downloaded from on July 31, 2017 - Published by www.plantphysiol.org Copyright © 2010 American Society of Plant Biologists. All rights reserved.
The widespread use of inappropriate agricultural practices (such as the excessive utilization of fungicides with elevated Cu concentrations), the production of industrial wastewaters, and mining activities have caused growing Cu contamination of cultivating soils and irrigating waters (He et al., 2005). Symptoms of excess Cu in plants include chlorosis, fewer and smaller leaves, diminished root growth, and flowering and germination defects (Marschner, 2002). When the environmental Cu concentration increases, Arabidopsis plants activate overlapping strategies that prevent the accumulation of damaging levels of Cu.

One of the mechanisms that Arabidopsis plants activate in response to the increase in environmental Cu consists of the transcriptional down-regulation of the expression of the high-affinity Cu transporter genes COPT1 and COPT2 (Sancenon et al., 2003). Recent evidence indicates that the SPL7 (for SQUAMOSA promoter-binding-like) transcription factor activates the expression of the COPT1 and COPT2 transporter genes in response to Cu deficiency, probably through binding to the GTAC motifs within their promoter regions (Yamasaki et al., 2009). Other SPL7 target genes induced upon Cu limitation include iron (Fe) superoxide dismutase FSD1 and microRNA miR398, which mediates Cu/zinc (Zn) superoxide dismutases, CSD1 and CSD2, mRNA down-regulation (Abdel-Ghany et al., 2005; Yamasaki et al., 2007, 2009). The substitution of CSD1 and CSD2 for its Fe counterpart, FSD1, is considered a hallmark strategy for replacing the dispensable Cu proteins by other metalloproteins, which, by using a different metal (usually Fe), perform a similar task. This strategy probably aims to save Cu for essential cuproproteins, such as plastocyanin, when scarce (Yamasaki et al., 2007). Cu deficiency mostly affects the plant reproductive structures of higher plants, which might be considered the most susceptible plant parts to Cu limitation (Graham, 1975; Marschner, 2002). This fact has also been corroborated in transgenic plants defective in Cu acquisition (Sancenon et al., 2004).

Mineral nutritional status, specifically an organic nitrogen metabolite, has been recently reported to affect the circadian clock in Arabidopsis (Gutierrez et al., 2008). A wide variety of biological processes are subjected to circadian clock control that translates environmental, light and temperature, and endogenous cycles into integrated temporal signals to rhythmically schedule physiology and metabolism at the adequate timing. Oscillations in gene expression, protein synthesis and degradation, nucleo/cytoplasmic partitioning, and protein phosphorylation are at the molecular base of the biological rhythms (for review, see McClung, 2006; Harmer, 2009; Mas and Yanovsky, 2009). Among the main central clock components in Arabidopsis are the single Myb domain transcription factors CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY; for review, see McClung, 2006; Harmer, 2009; Mas and Yanovsky, 2009). The LHY and CCA1 proteins bind to the same region of their target promoters, form homodimers or heterodimers, and colocalize within the nucleus, where they function synergistically (Lu et al., 2009). The loss-of-function double mutants lhy cca1 show short periods of free-running circadian rhythms, affected photoperiodic control of flowering time, modified hypocotyl length, and reduced plant height (Mizoguchi et al., 2002, 2005; Ito et al., 2007; Niwa et al., 2007; Lu et al., 2009). Plants whose clock period matches to the environmental cycles show photosynthetic and competitive advantages (Dodd et al., 2005). The transcriptional feedback loops have been shown to be interdependent of cytosolic rhythms, such as those of Ca$^{2+}$ and cyclic ADP-Rib, leading to the consideration of the circadian system as an integral part of the entire cell signaling network, which optimizes plant responses to the environment (Hastings et al., 2008; Harmer, 2009).

In this work, we describe how the COPT1 Cu transport protein localizes to the plasma membrane of Arabidopsis cells. Moreover, we show that the overexpression of the functional epitope-tagged COPT1 and COPT3 proteins in Arabidopsis causes an increase in endogenous Cu and that some of their phenotypes are reminiscent of altered circadian rhythms, such as those observed previously for plants defective in LHY and CCA1. Accordingly, expression analyses indicate that LHY and CCA1 mRNA levels significantly decrease as the endogenous content of Cu in Arabidopsis increases, which suggests that Cu homeostasis could affect, among other processes, the cellular circadian system.

**RESULTS**

**The COPT1 Cu Transport Protein Localizes to the Plasma Membrane of Arabidopsis Cells**

Although previous results indicate that the Arabidopsis COPT1 protein could function in Cu acquisition in the plasma membrane of specific plant cells (Kampfenkel et al., 1995; Sancenon et al., 2003, 2004), the fact that certain CTR members are located at intracellular endosomal vesicles or vacuolar membranes in other eukaryotic organisms (Bellemare et al., 2002; Rees et al., 2004; van den Berghe et al., 2007; Bertinato et al., 2008) prompted us to address the subcellular localization of COPT1 in Arabidopsis cells. To that end, the coding sequence of the COPT1 protein was fused to GFP (COPT1-GFP) under the control of
the constitutive cauliflower mosaic virus (CaMV) 35S promoter. Isolated Arabidopsis protoplasts transiently transformed with the COPT1-GFP construct were analyzed for green and red fluorescence, which are indicative of GFP and chlorophyll localization, respectively. Nontransformed protoplasts and the protoplasts transformed with a construct expressing only GFP were used as the negative and cytosolic positive controls, respectively (Fig. 1). The negative control shows a signal in the chloroplasts that presents high endogenous fluorescence due to chlorophyll (Fig. 1). In addition to the autofluorescence of chloroplasts, the COPT1-GFP-expressing cells display a specific signal on the cell surface that is absent in vacuoles, cytosol, and other discrete subcellular localizations (Fig. 1). The lypophilic styryl dye FM4-64, which becomes highly fluorescent upon binding to membranes, has been used at low temperatures as a marker to assess the plasma membrane colocalization with COPT1-GFP protein in Arabidopsis cells (Supplemental Fig. S1).

**Arabidopsis Seedlings Overexpressing COPT1 or COPT3 Display Increased Endogenous Cu Content**

To further understand the effect of deregulated Cu homeostasis on plant growth, transgenic plants overproducing either COPT1 or COPT3 were obtained. The coding sequences of the COPT1 and COPT3 transport proteins were tagged at their C-terminal regions with the human influenza virus hemagglutinin HA epitope (COPT1-HA and COPT3-HA constructs) to monitor protein levels. A heterologous complementation strategy with a ctr1Δctr3Δ yeast mutant strain, which is unable to grow on a medium containing glycerol as the only carbon source, has been used to test whether the COPT1-HA and COPT3-HA fusion proteins maintain their function in Cu transport in vivo. Yeast growth is restored when Glc or 100 μM CuSO₄ is added to the medium, indicating that the ctr1Δctr3Δ growth defect on glycerol is a respiratory defect as a result of decreased Cu delivery to the mitochondrial cytochrome oxidase (Fig. 2A). The lack of growth on the glycerol medium was restored when the yeast CTR1 or Arabidopsis COPT1 or COPT3 gene was constitutively expressed in the yeast mutant strain (Fig. 2A). Importantly, the expression of the COPT1-HA and COPT3-HA fusion proteins complemented the growth defect of the ctr1Δctr3Δ cells on glycerol similar to their untagged protein versions, indicating that the HA epitope does not interfere with the function of COPT1 and COPT3 in Cu transport in yeast. Once the functionality of the COPT1-HA and COPT3-HA fusion proteins had been verified, their coding sequences were cloned under the control of the constitutive CaMV35S promoter, and the CaMV35S:COPT1-HA and CaMV35S:COPT3-HA constructs were introduced by Agrobacterium tumefaciens transformation into the wild-type ecotype Columbia Arabidopsis plants (C1OE and C3OE plants). Several Arabidopsis transformed lines were analyzed by western blot using an antibody against the HA epitope. The lines with the highest levels of COPT1-HA and COPT3-HA protein (C1OE #1 and C3OE #3) were selected for further studies (Fig. 2, B and C).

We first analyzed the expression of COPT1 and COPT3 mRNAs in the wild type and the C1OE and C3OE seedlings grown on half-strength Murashige and Skoog medium (MS) or on the same medium supplemented with 10 μM CuSO₄. Consistent with their overexpression, both mRNA levels significantly increased in the C1OE and C3OE lines compared with the wild-type seedlings grown on MS, and their expression remained high on 10 μM CuSO₄ (Fig. 3, A and B). The C1OE seedlings displayed a 2.5-fold increase in the COPT1 mRNA expression on MS and a 3.5-fold increase on Cu medium, as compared with the wild-type

---

**Figure 1.** Arabidopsis COPT1 protein localizes to the plasma membrane. Arabidopsis protoplasts transiently transformed with the COPT1-GFP construct and analyzed by confocal microscopy 16 h after transformation are shown. The nontransformed protoplasts were used as a negative control, while the protoplasts transformed with a plasmid expressing GFP alone were used as a positive control. Green and magenta fluorescences are indicative of GFP protein and chlorophyll localization, respectively. Images show representative protoplasts at the same scale, including the merges and the light fields.
plants (Fig. 3A). Given the low levels of the COPT3 expression in the wild-type plants (Fig. 3B) and the different oligonucleotide efficiencies in the real-time reverse transcription (RT)-PCR assays, it is hard to assess COPT3 fold induction and to make comparisons with the COPT1 levels. Because the Arabidopsis COPT1 and COPT3 proteins have been implicated in Cu transport (Sancenon et al., 2004), we ascertained whether the overexpression of COPT1-HA and COPT3-HA alters the total content of Cu in the plant.

A moderate increase in seedling Cu content was observed for the C1OE and C3OE lines under both the MS and 10 μM CuSO₄ conditions (Fig. 3C). We further analyzed the C1OE and C3OE plants by determining the mRNA levels of several genes regulated by Cu status. This analysis included the high-affinity Cu transporter COPT2 gene and the Fe superoxide dismutase FSD1 gene, whose expression is up-regulated upon Cu limitation, as well as the Cu/Zn superoxide dismutase CSD1 and CSD2 genes, which display the opposite

Figure 2. Obtaining and selection of COPT1- and COPT3-overexpressing Arabidopsis plants. A, Complementation of the growth phenotype of the S. cerevisiae ctr1Δctr3Δ mutant by the COPT1-HA and COPT3-HA constructs. Yeast cells transformed with an empty vector (p426GPD, negative control), a vector containing yeast CTR1 or Arabidopsis COPT1 and COPT3 (positive controls), or the COPT1-HA and COPT3-HA constructs were assayed for growth on Glc, glycerol, or glycerol plus 100 μM CuSO₄ iCu. Cells were spotted in 10-fold serial dilutions starting at A₆₀₀ = 0.1. B, Detection of COPT1-HA-fused protein in the transgenic plants. Western-blot analysis of the total protein extracts from leaves of the wild type (WT) and five independent transgenic C1OE lines. C, Detection of COPT3-HA-fused protein in the transgenic plants. Western-blot analysis of the total protein extracts from leaves of the wild type and five independent transgenic C3OE lines. The western blots shown in B and C were developed with commercial antibody against the HA epitope.

Figure 3. Expression levels and endogenous Cu contents in COPT1- and COPT3-overexpressing Arabidopsis seedlings. A, Quantification by real-time RT-PCR of the COPT1 mRNA expression levels from the wild-type (WT; plain bars) and C1OE (striped bars) seedlings grown on MS plates either without (MS; white bars) or with 10 μM CuSO₄ (Cu; gray bars) under 6 neutral days with photocycle and thermocycle. The UBQ10 gene was used as a loading control. Values are means ± sd of at least three technical replicates. a.u., Arbitrary units; R.E., relative expression. B, Quantification by real-time RT-PCR of the COPT3 mRNA expression levels, as indicated in A, except C3OE (dotted bars). C, Cu content from the seedlings shown in A and B. Values are means ± sd of at least three technical replicates. D.W., Dry weight. Asterisks indicate statistically significant differences with respect to the wild type (* P < 0.05, ** P < 0.01).
pattern of expression, that is, they are down-regulated upon Cu limitation (Abdel-Ghany et al., 2005). Consistent with the increase noted in the endogenous Cu content, the C1OE and C3OE plants displayed an increase of CSD1 and CSD2 mRNAs along with a decrease of the COPT2 and FSD1 transcript levels (Supplemental Fig. S2). These combined results demonstrate that the Arabidopsis plants overexpressing epitope-tagged versions of the COPT1 and COPT3 transport proteins display increased endogenous Cu levels, which are perceived by the plant Cu sensor system.

**COPT-Overexpressing Arabidopsis Seedlings Are Sensitive to Elevated Cu Concentrations in the Environment**

The phenotypic analysis of seed germination and seedling growth on plates revealed that the C1OE and C3OE seedlings did not appreciably differ from the wild-type ones when they were grown on MS. In the presence of 10 μM CuSO4, however, the growth of the C1OE and C3OE seedlings was severely affected. More specifically, the C1OE and C3OE seedlings grown on 10 μM CuSO4 displayed a dramatic inhibition in root growth, and additional lateral roots emerged near the crown (Fig. 4A). Furthermore, the growth of the C1OE and C3OE seedlings was impaired as the Cu concentration in the growth medium increased (data not shown). To accurately quantify the sensitivity of the C1OE and C3OE plants to environmental Cu, the root length of the wild-type, C1OE, and C3OE seedlings was measured in the presence of increasing concentrations of Cu in the medium.

As shown in Figure 4B, whereas wild-type root growth remained unaffected by concentrations up to 20 μM CuSO4, the root length of the C1OE and C3OE seedlings drastically shortened at a concentration of 10 μM CuSO4. Complete root growth inhibition was observed for the C1OE and C3OE seedlings grown on MS supplemented with 50 μM CuSO4 (Fig. 4B). To ascertain whether transgenic seedling sensitivity to Cu was specific, different metal salts were added to the growth medium and fresh weight was determined for the C1OE seedlings. As Figure 4C depicts, the addition of 30 μM CuSO4 to MS lowered the fresh weight of the C1OE seedlings three times. However, a decrease in fresh weight as drastic as the one observed by Cu treatment was not observed when other metals (Fe, Zn, and cadmium) were added to the medium as sulfates at a concentration of 30 μM (Fig. 4C). Only silver (Ag), whose Ag+ ion is isoelectric to Cu+, led to a significant decrease in fresh weight (Fig. 4C). Taken together, these results indicate that the Arabidopsis seedlings overexpressing COPT1 are specifically sensitive to Cu in the growth medium, and they highlight the need for a tight control of Cu homeostasis in higher plants.

**Additional Phenotypes Associated with the COPT-Overexpressing Arabidopsis Plants**

To further address the effects of the altered regulation of Cu homeostasis on plant physiology, we stud-
ied the fairly similar phenotypes observed for the C1OE and C3OE plants grown on soil. The global size of the C1OE and C3OE plants was substantially smaller compared with the wild-type ones (Fig. 5A; Supplemental Fig. S3A). Both the 4-week-old C1OE and C3OE plants grown on soil were around half the size of the wild-type plants (Table I). A closer view of the C1OE and C3OE plants revealed that the first pair of rosette leaves displayed a perpendicular orientation to the normal plane (Fig. 5B; Supplemental Fig. S3B). Moreover, the C1OE leaves presented an adaxial curvature instead of the abaxial curvature observed in the wild-type leaves (Fig. 5C). Reproductive organs in the C1OE and C3OE plants were also affected, as most flowers presented short petals and stamens protected by the sepals throughout flower development (Fig. 5D; Supplemental Fig. S3C). This fact could account for the low fertility observed in the C1OE plant lines (data not shown). Furthermore, the flowering time of the C1OE and C3OE plants was slightly delayed in long-day conditions compared with the wild-type plants, as indicated by a higher leaf number at the time of flowering. Yet, the flowering time inverted in short-day conditions and was somewhat advanced in the C1OE and C3OE plants (Table I). Moreover, the COPT-overexpressing hypocotyls were longer than the wild-type ones in white light, but the C1OE hypocotyls were shorter than the controls in both darkness and continuous red light (Table I). These additional phenotypes further highlight the importance of the spatiotemporal control of Cu homeostasis to ensure appropriate growth and development in higher plants. It is noteworthy that none of these phenotypes was observed when the Arabidopsis wild-type plants were grown on soil with elevated Cu levels, although some of them were rather reminiscent of plants with altered circadian rhythms (Mizoguchi et al., 2002, 2005; Ito et al., 2007; Niwa et al., 2007; Lu et al., 2009), suggesting potential alterations in the perception of light or circadian rhythms in the plants where Cu homeostasis is deregulated.

Given that light and temperature are classical environmental entrainment factors of the plant circadian rhythms, we tested the effect of the absence of light and temperature cycles on the development of the C1OE and C3OE plants. Both transgenic lines were grown on MS plates under neutral days with a synchronized photocycle and thermocycle (LDHC), or with either the photocycle (LLHC) or the thermocycle (LDHH) absent, or with both absent (LLHH). Figure 6 and Supplemental Figure S4 show the data obtained under the LDHC and LLHH conditions, whereas Supplemental Figure S5 presents the data obtained under the LLHC and LDHH conditions. Figure 6A and Supplemental Figure S4A show the seedlings grown for 6 d on MS plates either with or without 10 μM CuSO4. The growth of the C1OE and C3OE seedlings was more affected than that of the wild-type controls given the absence of environmental cycles; accordingly, the fresh weight per seedling further decreased (Fig. 6B). As stated, the root length of the C1OE and C3OE seedlings grown under LDHC conditions was greatly reduced by 10 μM CuSO4 compared with the wild type (Fig. 4B). In the absence of environmental cycles, however, the root length notably decreased.
in the COPT-overexpressing seedlings, even on MS (Supplemental Fig. S4), which further indicates a connection between Cu transport and daily cycles.

In order to address whether the difficulties of the C1OE and C3OE seedlings to thrive in the absence of environmental inputs were caused by photooxidative damage, we determined total chlorophylls and anthocyanins as well as lipid peroxidation, measured as malondialdehyde (MDA) content, under the different photocycle and thermocycle conditions (Fig. 6; Supplemental Fig. S5). When plants were grown under LLHH conditions and were compared with the LDHC conditions, a decrease in MDA content (Fig. 6D) was accompanied by an increase in the total chlorophyll and anthocyanin contents (Fig. 6, C and E). However, no consistent differences in MDA and the chlorophyll or anthocyanin levels were observed among the wild-type and the COPT-overexpressing seedlings on MS and subjected to LDHC conditions, a decrease in anthocyanins (Supplemental Fig. S5E; LLHC) was only detected under continuous conditions (Fig. 6; Supplemental Fig. S5). Taken together, these data prompted us to analyze the expression of the circadian clock components LHY and CCA1. Samples were harvested at dawn, Zeitgeber time 0 h, when the mRNA levels of LHY and CCA1 show a peak of expression (Mizoguchi et al., 2002). As shown in Figure 7, the C1OE and C3OE seedlings grown on MS and subjected to LDHC conditions exhibited a significantly reduced LHY and CCA1 expression when compared with the wild-type ones. As a result of this reduction, it is expected that circadian clock outputs, such as the expression of CONSTANS-LIKE1 (COL1; Mizoguchi et al., 2002) and LHCBI.1 (Salomé et al., 2008) genes, should also be altered in the C1OE and C3OE plants. In addition to LHY and CCA1, the expression levels of the COL1 and LHCBI.1, on both the MS and 10 μM CuSO4, indeed decreased if compared with the wild type (Fig. 7).

In order to further assess the role of Cu in the expression of the LHY and CCA1 genes, the wild-type seedlings were grown on MS and 10 μM CuSO4 under LDHC conditions, and the mRNA levels were analyzed in two diurnal cycles (Fig. 8A). The presence of Cu in the medium slightly decreased LHY mRNA but CCA1 mRNA was reduced to about 50%, affecting the amplitude of their oscillations (Fig. 8A). When the LHY and CCA1 expression was analyzed at dawn (time for the peak of LHY and CCA1 expression) in the wild-type seedlings grown at different Cu concentrations, we observed that LHY and CCA1 expression levels lowered in a dose-dependent manner (Fig. 8B). The effect of Cu on the expression of these genes, particularly CCA1, is comparable to the regulation observed for the well-established Cu-regulated gene COPT2 (Fig. 8B).

To ascertain whether Cu affects the circadian expression of the circadian clock components, the luciferase activity of the PLHY:Luc seedlings, which fully recapitulate the rhythmic pattern of the expression of the endogenous LHY transcript (Kim et al., 2003), was...
monitored at different environmental Cu statuses. After a 7-d entrainment to the LDHC cycles, the PLHY:luc seedlings were then released into either continuous light or dark at a constant temperature (25°C). The analysis of the three luciferase activity cycles after being transferred into free-running conditions is shown either under continous light (Fig. 9A) or in the dark (Fig. 9B). Cu down-regulated the LHY expression by affecting the peak amplitude, which decreased (Fig. 9). Error bars are not included in this figure for simplicity, but as Supplemental Figure S6A shows, the SE of the mean of the MS and 10 µM CuSO₄ data do not overlap at the oscillation peaks. Changes in the period, amplitude, and phase of the oscillations shown in Figure 9 and Supplemental Figure S6 have been estimated and statistically analyzed (Supplemental Table S1). Whereas the LHY expression oscillation period remained unchanged after being transferred into free-running conditions, the amplitude significantly decreased (Supplemental Table S1; Supplemental Fig. S6A) and the phase was delayed (Supplemental Table S1; Supplemental Fig. S6A) on Cu medium compared with seedlings grown on MS. Moreover, the PLHY:luc seedlings, which had been entrained for 7 d to the LDHC cycles and transferred to continuous light, were subjected to Cu addition at different circadian oscillation phases (Supplemental Fig. S6B). Irrespective of the addition time, Cu mod-
ified the amplitude and the phase of the circadian LHY expression oscillation to a similar extent but did not affect its period (Supplemental Table S1; Supplemental Fig. S6B). Furthermore, when the PLHY:Luc seedlings growing on plates with 10 μM CuSO₄ were subjected to seven LDHC cycles and were then transferred to free-running light conditions in the presence of the specific Cu chelator bathocuproine disulfonic acid, the amplitude of oscillation increased, indicating the reversibility of the Cu effect (Supplemental Fig. S6C).

In order to further assess the role of Cu in the circadian clock, and in addition to LHY and CCA1, the expression of other genes considered to be outputs of the clock, such as COL1 and LHCBl1.1, was analyzed by real-time RT-PCR under free-running light conditions.

Seedlings were grown over a 6-d period with LDHC cycles. After being transferred to continuous light conditions, samples were taken at Zeitgeber time 0 h during the subjective day in cycles 2 (LC2) and 3 (LC3). The results shown in Figure 10 reflect that Cu also down-regulates the clock output genes COL1 and LHCBl1.1.

Taken together, these results indicate that deregulated Cu homeostasis in COPT-overexpressing plants is detrimental for their growth, especially under continuous environmental conditions. One of the processes affected by Cu is the expression of the main components of the circadian clock LHY and CCA1 genes and their outputs.

Figure 7. Regulation of the gene expression of circadian clock components and clock output genes in the COPT1- and COPT3-overexpressing Arabidopsis seedlings. Quantification by real-time RT-PCR of the CCA1, LHY, LHCBl1.1, and COL1 mRNA expression levels from the wild-type (WT; plain bars), C1OE (striped bars), and C3OE (dotted bars) seedlings grown on MS plates either without (MS; white bars) or with 10 μM CuSO₄ (Cu; gray bars) under 6 neutral days with photocycle and thermocycle. Samples were taken at Zeitgeber time 0 h. The UBQ10 gene was used as a loading control. Values are means ± SD of three technical replicates. a.u., Arbitrary units; R.E., relative expression. Asterisks indicate statistically significant differences with respect to the wild type (* P < 0.05, ** P < 0.01).

Figure 8. Cu regulation of the circadian clock and Cu homeostasis genes in Arabidopsis seedlings. A, RT-PCR products from total RNA of the wild-type seedlings grown on MS plates either without (MS; white circles) or with 10 μM CuSO₄ (Cu; black squares) under 6 neutral days with photocycle and thermocycle. Samples were taken every 4 h during the following 48 h. The relative intensity of the electrophoretic band for each gene versus the 18S band, used as a loading control, is represented. The experiments were repeated at least three times with similar results, and a representative experiment is shown. ZT, Zeitgeber time. The white and black bars at bottom indicate day and night, respectively. a.u., Arbitrary units; R.E., relative expression. B, RT-PCR products from total RNA of wild-type seedlings grown on MS plates with 0.2, 1, 10, or 50 μM CuSO₄ under 6 neutral days with photocycle and thermocycle. Samples were taken at Zeitgeber time 0 h. 18S rRNA was used as a loading control.
DISCUSSION

The existence of tightly regulated Cu homeostasis networks in plants has been traditionally attributed to not only ensure Cu delivery to essential cuproproteins but to also avoid harmful effects of Cu when in excess. Despite this, very little is known about the specific effects of deregulated Cu homeostasis on plant physiology. Previous works have shown that Arabidopsis plants express a family of CTR-type Cu transport proteins, know as COPT1 to -6 (Kampfenkel et al., 1995; Sancenon et al., 2003, 2004), and the COPT1 protein has been shown to function in plant Cu acquisition (Sancenon et al., 2004). Here, we ascertained COPT1 subcellular localization by expressing a GFP-tagged COPT1 protein in Arabidopsis protoplasts. Similar to other eukaryotic high-affinity Cu transporters of the CTR family, the COPT1 protein localizes to the plasma membrane of Arabidopsis cells (Fig. 1; Supplemental Fig. S1), which is not only consistent with its function in plant Cu acquisition but further reinforces its role in the cellular Cu uptake in Arabidopsis cells.

Several members of the Arabidopsis COPT family, including COPT1 and COPT2, are transcriptionally down-regulated by Cu, whereas others, like COPT3 and COPT5, are not regulated by Cu (Sancenon et al., 2003). Both the COPT-mediated Cu transport activity and the Cu-inhibited expression of the COPT genes integrate a negative autoregulatory feedback loop (Péanarrubia et al., 2010). In order to gain further insight into the specific role of Cu homeostasis in plant growth and plant development, we overexpressed the COPT1 and COPT3 genes in Arabidopsis driven by the constitutive CaMV35S promoter. Therefore, with the substitution of the native COPT1 and COPT3 regulatory regions for the constitutive CaMV35S promoter, we would expect not only the increased and ectopic expression of the genes but also the loss of the negative autoregulatory feedback loop. Consequently, the C1OE and C3OE plants should display an increased and constitutive Cu transport activity toward the cytosol in most tissues. To easily determine the COPT1 and COPT3 protein levels and to select suitable lines, we tagged their protein sequences with the HA epitope at the C-terminal regions. Notably, the HA tag did not significantly interfere with the COPT1 and COPT3 protein functions in Cu transport, as shown by functional complementation experiments in yeast (Fig. 2A). However, we cannot rule out that the HA epitope tag alters putative COPT1 posttranslational regulatory mechanisms, including Cu-induced endocytosis, degradation, and transport blockage, which have been previously described for other eukaryotic high-affinity Cu transporters of the CTR family (Ooi et al., 1996; Petris et al., 2003; Guo et al., 2004; Liu et al., 2007). In this sense, although the Cys residues at the yeast Ctr1 C-terminus are dispensable for Cu transport, they have been postulated to serve as Cu sensors and to play a crucial role in preventing Cu uptake when intracellular Cu levels rise (Wu et al., 2009). Indeed, the lack of this regulatory mechanism in yeast cells expressing either a Ctr1 protein with an epitope tag or a deletion at its C terminus leads to sensitivity to Cu (Wu et al., 2009). Therefore, the C1OE and C3OE plants represent not only an increase but a modification in the Cu uptake pattern, which probably affects the regulation of COPT1 and COPT3 at the transcriptional and post-translational levels and which would modify the content and the spatiotemporal distribution of the Cu transport in these plants.
Figure 10. Cu regulation of the gene expression of circadian clock components and clock output genes in the Arabidopsis seedlings. A, Quantification by real-time RT-PCR of the CCA1, LHY, LHCBI.1, and COL1 mRNA expression levels from wild-type seedlings grown on MS plates either without (MS; white bars) or with 10 μM CuSO4 (Cu; gray bars), entrained under 6 neutral days with photocycle and thermocycle, and transferred to continuous light. Samples were taken at Zeitgeber time 0 h on the subjective day for continuous light cycle 2 (LC2) and cycle 3 (LC3) after transferring. The UBQ10 gene was used as a loading control. Values are technical replicate means ± sd (n ≥ 6) from two independent experiments. a.u., Arbitrary units; R.E., relative expression. Asterisks indicate statistically significant differences with respect to the wild type (* P < 0.05, ** P < 0.01).

Given the elevated specificity of the COPT transport proteins for the Cu+ ion (Sancenon et al., 2003, 2004), the phenotypes observed for the C1OE and C3OE plants should be highly Cu specific. In this sense, the C1OE and C3OE lines are an excellent tool to study the effects of deregulated Cu transport on plant physiology. As expected, the C1OE and C3OE plants display a moderate increase in endogenous Cu levels under both low and high Cu conditions in the medium when compared with the wild-type plants (Fig. 3C). Notably, endogenous Cu in the C1OE and C3OE plants is biologically active and perceived by the Cu transcriptional factor SPL7, as suggested by the changes in the expression of the well-known Cu status markers (FSD1 and COPT2 for low Cu and CSD1 and CSD2 for high Cu; Supplemental Fig. S2; Abdel-Ghany et al., 2005, Yamasaki et al., 2009). Root length is a highly sensitive parameter that is frequently used to test plant Cu sensitivity (Sancenon et al., 2004; Andrés-Colás et al., 2006). Hence, the C1OE and C3OE lines are more sensitive to the presence of Cu in the growth medium than the wild-type plants (Fig. 4, A and B). Cu specificity is reflected in the decreased fresh weight of the C1OE plants, which is selectively caused by Cu+ and Ag+, an isoelectric element to Cu+ that can partially mimic the effects of Cu (Fig. 4C).

On the other hand, it is interesting to highlight that the phenotypes observed in the C1OE and C3OE plants are more exacerbated than expected by the endogenous Cu content of these plants. So, whereas the growth of the C1OE and C3OE seedlings on MS (endogenous Cu content around 6 μg g−1 dry weight) is affected under continuous environmental conditions (Fig. 6B; LLHH, compare C1OE and C3OE with the wild type on MS), the growth of the wild-type seedlings on 10 μM CuSO4 (endogenous Cu content around 32 μg g−1 dry weight) remains mostly unaffected (Fig. 6B; LLHH, compare the wild type on MS and Cu). Furthermore, despite the endogenous Cu content of the wild-type seedlings in the 10 μM CuSO4 medium being around 32 μg g−1 dry weight and approximately 45 μg g−1 dry weight in the C1OE and C3OE seedlings (Fig. 3C), changes in gene expression (Supplemental Fig. S2), and particularly the visual phenotypes displayed by the C1OE and C3OE seedlings (Fig. 5; Table I; Supplemental Fig. S3), are not observed in the wild-type seedlings grown on 20 to 25 μM CuSO4, despite their endogenous Cu content being around 100 μg g−1 dry weight (data not shown). These results indicate that the observed phenotypic effects in the C1OE and C3OE seedlings are not solely due to their endogenous Cu content but are a result of the ectopic and deregulated Cu transport in these plants, which could drive the difficulty of C1OE and C3OE plants to thrive, especially in the absence of environmental inputs. Neither lipid peroxidation nor the anthocyanin content measured in the C1OE and C3OE seedlings indicates a drastic increase in the oxidative conditions of these plants compared with the wild-type ones (Fig. 6, D and E; Supplemental Fig. S5, D and E), further suggesting additional alterations caused by deregulated Cu homeostasis, where environmental light and temperature cycles are involved.

Interestingly, some of the phenotypes displayed by the C1OE and C3OE plants, including the number of rosette leaves at the reproductive transition and hypocotyl length (Table I), are reminiscent of those obtained for the loss-of-function double mutant lhy cca1, which are nuclear components of the Arabidopsis circadian clock (Mizoguchi et al., 2002, 2005; Ito et al., 2007; Niwa et al., 2007). Accordingly, the C1OE and C3OE plants show decreased expression levels of the LHY and CCA1 genes and of the circadian clock output genes LHCBI.1 and COL1 when compared with the...
wild-type plants (Fig. 7). These data show that the increased and deregulated Cu transport occurring in the C1OE and C3OE plants modifies the expression of at least two crucial transcription factors that influence Arabidopsis chronobiology. Consequently, the results shown here indicate that the increased metal uptake strategies that aim to hyperaccumulate metals should take into account that deregulated metal homeostasis can affect other general and maybe interconnected cellular processes, such as the circadian system, as shown in this work. Nevertheless, the altered expression of circadian clock components cannot justify by itself the observed phenotypes in COPT-overexpressing plants grown on soil, since Arabidopsis mutants affected in circadian clock components (Dodd et al., 2005; McClung, 2006) do not display all the phenotypes described in this work (Figs. 4 and 5; Supplemental Fig. S3). These phenotypes could rather account for the Cu toxic effects when its transport is deregulated.

As a first approach to gain further insight into the Cu influence on LHY and CCA1 expression, their daily expression patterns have been shown to be Cu responsive (Fig. 8). Furthermore, the luciferase activity of the transgenic PLHY:Luc plants, grown for 7 neutral days and then subjected to continuous light or dark, also reveals that the amplitude of the LHY circadian expression is Cu responsive (Fig. 9B; Supplemental Table S1; Supplemental Fig. S6A). However, Cu has no effect on the LHY circadian oscillation period under the different conditions studied (Fig. 9; Supplemental Table S1; Supplemental Fig. S6), and the LHY oscillation phase is delayed by Cu (Supplemental Table S1; Supplemental Fig. S6A). Moreover, real-time RT-PCR corroborates that the wild-type seedlings display decreased levels of the Myb transcription factors LHY and CCA1 and the circadian clock targets LHCBI.1 and COL1 mRNAs under Cu treatment (Fig. 10).

In addition to the role of the SPL7 transcription factor in Cu deficiency responses, other Arabidopsis SPL members have been shown to participate in flowering transition and fertility (Cardon et al., 1999; Unle et al., 2003; Wu and Poethig, 2006; Gandikota et al., 2007; Zhang et al., 2007; Wang et al., 2009; Wu et al., 2009). It is interesting that two members of the Physcomitrella patens SPL family, which are homologous to Arabidopsis SPL8, are regulated by the circadian clock (Rieze et al., 2008). Furthermore, the AtSPL7 expression cycles under the different diurnal and circadian conditions, as indicated in the data available in the Diurnal Oregon State University Database (http://diurnal.cgrb.oregonstate.edu/). Moreover, the AtSPL7 target, miR398, displays a diurnal oscillatory pattern of accumulation (Sire et al., 2009). The SPL genes present GTAC motifs within their promoters at a higher occurrence than in the randomly selected promoters, which have been shown to mediate the autoregulation by possibly using a feedback loop based on protein interactions and transcriptional regulation (Wang et al., 2009). A single GTAC motif has recently been shown to be sufficient to confer the Cu responsiveness mediated by SPL7 to the miR398 promoter in Arabidopsis (Yamasaki et al., 2009). A bioinformatic search within the promoters of circadian clock genes performed with the PLACE Web Signal Scan (http://www.dna.aaffrc.go.jp/htdocs/PLACE/signalscan.html) and the Patmatch program from The Arabidopsis Information Resource (www.arabidopsis.org) was performed (data not shown). The presence of a GTAC motif within the LHY (−160 to −156 bp distance to the translational start site) and the CCA1 (−132 to −128) 5′ untranslated regions suggests a possible explanation of how Cu influences the expression of the central oscillator components by down-regulating their expression via SPL7. Although the regulatory mechanism is far from being elucidated and further studies are necessary to understand the mechanisms that underlie the influence of Cu on the circadian clock in higher plants, taken together, these data suggest that members of the complex Arabidopsis SPL family of transcription factors may act as key integrators between Cu status and circadian responses in plants.

Chloroplasts indeed play a crucial role in the connection between metal homeostasis and the circadian clock. First, many critical metal-dependent processes take place in chloroplasts that constitute a major subcellular transition metal sink, where plastocyanin and Cu/Zn superoxide dismutase are the main chloroplastic cuproproteins. During chloroplast biogenesis, light and tissue signals promote the nuclear-chloroplastic coordinated synthesis of hundreds of chloroplast proteins, including different metalloproteins (López-Juez, 2007). Presumably, metal requirements should be higher during the diurnal light phase in chloroplasts. Although little is known about the master switch regulators in the chloroplast biogenesis, the challenges that subcellular metal trafficking pose to the daily light/dark changes under intracellular redox conditions are emerging as a determinant process to fully understand dynamic aspects in transition metal homeostasis (Puig and Peñarrubia, 2009). In this sense and under Cu deficiency, the chloroplastic Cu/Zn superoxide dismutase is substituted by the Fe superoxide dismutase (Abdel-Ghany et al., 2005), which has already been shown to display a circadian expression pattern (Kliebenstein et al., 1998). Interestingly, it has been recently shown that the regulation of the chloroplastic Fe storage protein, ferritin, is an output of the central oscillator (Duc et al., 2009); thus further suggesting that metal homeostasis is subjected to the circadian control in Arabidopsis. Furthermore, the different proteins that participate in plastid-to-nucleus retrograde signaling also affect the circadian clock (Hassidim et al., 2007; Larkin and Ruckle, 2008).

The results we present here indicate that other than playing a role to ensure Cu availability while avoiding toxic effects, tightly spatiotemporally regulated Cu homeostasis is further required for Arabidopsis proper fitness in the absence of environmental cycles.
The alterations in Cu homeostasis in the C1OE and C3OE plants down-regulate the circadian expression levels of the crucial circadian clock components in Arabidopsis. Thus, the control of Cu homeostasis, probably at both the spatial and temporal levels, could be important to fulfill the cell-specific and appropriate timing requirements of Cu daily intracellular traffic. In this sense, Cu homeostasis could participate in the integral cellular circadian system, possibly through the effect of Cu on the expression of circadian clock components, as shown in this work.

MATERIALS AND METHODS

Plant Growth Conditions and Treatments

Arabidopsis (Arabidopsis thaliana ecotype Columbia) plants were grown on soil as described previously (Andrés-Colás et al., 2006) for the indicated times. To grow seedlings on plates, seeds were surface sterilized, stratified for 2 d at 4°C, and incubated on MS with 1% Suc, either supplemented or not with CuSO4 for the indicated times. MS plates contain 0.1 μM CuSO4, which is considered a concentration below the optimal Cu supply (Abdel-Ghany et al., 2005; Yamakasi et al., 2009). Plants were grown under long-day (16 h of light-23°C/8 h of dark-16°C; LD), short-day (8 h of light-23°C/16 h of dark-16°C; SD), or neutral-day conditions with a synchronized photocycle and thermocycle (12 h of light-23°C/12 h of dark-16°C; LDHC) or in the absence of photocycle (LLHC), thermocycle (LDHH), or both (LLHH), as indicated. Fresh weight measurements were taken from five to 15 seedlings. Hypocotyl length was determined by the trichlorometric method (Parsons and Strickland, 1963).

Gene Expression Analysis by RT-PCR

Transgenic Plants Overexpressing COPT1 or COPT3

The complete COPT1 and COPT3 coding sequences were obtained from Arabidopsis genomic DNA by PCR using the following specific primers, which introduce the adequate restriction sites for cloning: COPT1-Xhol forward, 5'-GGCTCTAGACATGGATCATGATCACATGC-3'; COPT1-BglII reverse, 5'-GAGAATTCAGAAGCGCCATGAGTGGAC-3'; COPT1- Bg/II reverse, 5'-GAGAATTCAGAAGCGCCATGAGTGGAC-3'; COPT3-BglII reverse, 5'-GGCTCTAGACATGGATCATGATCACATGC-3'; COPT3-Bg/II reverse, 5'-GGCTCTAGACATGGATCATGATCACATGC-3'. The C terminus was tagged with the HA epitope through its insertion into the pPILY vector (Ferrando et al., 2000) with the Xhol and Bg/II restriction enzymes. The function of the tagged protein was checked by yeast complementation as described previously (Sancenon et al., 2003). The tagged protein was cloned under the control of the constitutive CaMV35S promoter into the binary pBII1 vector with the Xhol and Sal restriction enzymes. The C35S strain of Agrobacterium tumefaciens transformed with this construct was used to transform the Arabidopsis plants by following the floral dip protocol (Clough and Bent, 1998). Transgenic plants were selected by kanamycin resistance and by western blot analysis with the total protein extracts from the leaves of adult plants, using a commercial anti-HA antibody (3F10; Roche).

Gene Expression Analysis by RT-PCR

Supplemental Data

The following materials are available in the online version of this article.
COPT1 and COPT3 Overexpression Effects in Arabidopsis


Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carre IA, Coupland G (2002) LHY and CCA1 are partially redundant...
genes required to maintain circadian rhythms in Arabidopsis. Dev Cell 2: 629–641