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## Article Addendum

### Daily rhythmicity of high affinity copper transport

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of copper deficiency responses by diurnal and circadian rhythms in *Arabidopsis thaliana*. J Exp Bot; doi:10.1093/jxb/erv474.

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### Abstract

A differential demand for copper (Cu) of essential cupro-proteins that act within the mitochondrial and chloroplastal electronic transport chains occurs along the daily light/dark cycles. This requires a fine-tuned spatiotemporal regulation of Cu delivery, becoming especially relevant under non-optimal growth conditions. When scarce, Cu is imported through plasma membrane-bound high affinity Cu transporters (COPTs) whose coding genes are transcriptionally induced by the SPL7 transcription factor. Temporal homeostatic mechanisms are evidenced by the presence of multiple light- and clock-responsive regulatory *cis* elements in the promoters of both *SPL7* and its *COPT* targets. A model is presented here for such temporal regulation that is based on the synchrony between the basal oscillatory pattern of *SPL7* and its targets, such as *COPT2*. Conversely, Cu feeds back to coordinate intracellular Cu availability on the *SPL7*-dependent regulation of further Cu acquisition. This occurs via regulation at *COPT* transporters. Moreover, exogenous Cu affects several circadian-clock components, such as the timing of *GIGANTEA* transcript abundance. Together we propose that there is a dynamic response to Cu that is integrated over diurnal time to maximize metabolic efficiency under challenging conditions.

**Key words**

*Arabidopsis thaliana*; circadian clock; copper deficiency; copper transport; diurnal rhythm; heavy metals.

TEXT

### Plasma membrane COPTs and copper transport

Temporal ordering of biological processes and stress responses confer fitness advantages to plants.<sup>1, 2</sup> Response to the day-night cycle requires a coupled system of light perception and the circadian clock. Together this creates an environmentally set, endogenous timekeeper that appropriately schedules daily activities, including nutrient transport.<sup>3, 4</sup> Transition metals, such as copper (Cu), while being essential nutrients whose requirements vary along the day, are also potentially toxic during intracellular trafficking, owing to their ability to cause oxidative stress.<sup>5, 6</sup> Both features, essentiality and toxicity, require fine-tuned and narrow homeostatic mechanisms that, in the case of Cu, are evolutionary conserved.<sup>7</sup>

High affinity Cu transporters (COPTs, CTRs in organisms other than plants) are key components of the major pathway for cellular high-affinity Cu uptake. Three out of the six *Arabidopsis thaliana* COPT family members (COPT1, COPT2 and COPT6) are located at the plasma membrane.<sup>7</sup> Under Cu deficiency, increase in transcript levels of these three Cu transporters is driven by the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 (SPL7) transcription factor.<sup>8</sup> Among them, *COPT2* is the most highly expressed in young and adult tissues.<sup>9</sup>

Cu uptake depends on both its availability in the apoplast and the density of Cu transporter proteins at the plasma membrane.<sup>10</sup> Under deficiency, the predominant strategy for Cu acquisition consists in a mechanism of reduction of Cu<sup>2+</sup> to Cu<sup>+</sup> and subsequent Cu<sup>+</sup> uptake.<sup>11, 12</sup> Cu entrance is mostly dependent on the quantity of high affinity Cu transporters. Variations in external Cu concentrations in the deficiency range (below 0.5  $\mu\text{M}$ )<sup>13</sup> have little effect on Cu influx. This is because the  $K_m$  for the COPT2 transporter may be around 0.1  $\mu\text{M}$ , assuming a Cu affinity similar to that for the *Chlamydomonas* transporters.<sup>14</sup> Thus, under Michaelis-Menten kinetics ( $J_{in} = J_{max} \times [\text{Cu}] / (K_m + [\text{Cu}])$ ), the effect of doubling the external Cu concentration in the apoplast (from 0.2 to 0.4  $\mu\text{M}$  Cu,  $J_{0.4\text{Cu}}/J_{0.2\text{Cu}}$ ) at a constant COPT2 level would result in only a marginal 1.1 fold increase of the unidirectional Cu influx through the membrane. However, considering that the influx linearly responds to the number of transporters, the effect of doubling the presence of the COPT2 transporter at the plasma membrane ( $J_{2\text{COPT2}}/J_{1\text{COPT2}}$ ) at a fixed external Cu concentration would result in a two-fold increase. Thus, Cu influx is dependent on the number of specific high affinity Cu<sup>+</sup> transporters. The relevance of the COPT level in Cu uptake has been demonstrated in transgenic plants where *COPT1* expression has been altered from overexpressing to complete loss.<sup>10</sup> We conclude from these studies that the regulation of the expression of the COPT transporters has a strong effect on the Cu concentrations inside cells.

### Plasma membrane high affinity copper transport is under light and circadian control

The circadian clock participates in the regulation of nutrient transport<sup>3</sup> and stress responses<sup>1, 2</sup> and light affects the magnitude of the Cu-deficiency responses.<sup>15</sup> Transcriptional regulation of the encoded plasma-membrane high-affinity Cu transporter *COPT2* is subjected to both light and circadian control.<sup>15</sup> Thereby, basal *COPT2* expression oscillates under day/night cycles with a period of around 24 h, that is maintained even in the absence of environmental cycles (Figure 1). More specifically, *COPT2* expression peaks around 0 h (dawn), which coincides with rapid increase in Cu demand of the chloroplasts.<sup>15</sup> *COPT1* has also been shown to exhibit a daily oscillation regulated by the circadian clock.<sup>3</sup> According to the DIURNAL database,<sup>16</sup> *COPT1* expression peaks at 21.5 h, which is only several hours before the subsequent dawn. Furthermore, analysis of the *COPT1*, *COPT2* and *COPT6* promoter regions reveals multiple putative light and circadian *cis*-regulatory elements.<sup>9, 17, 18</sup> Some of these motifs are involved in phytochrome regulation of gene expression,<sup>19</sup> which corroborates the reduction of *COPT2* transcript in the *phy* mutants.<sup>15</sup> Such *cis*-responsive elements could also participate in the regulation of the transcriptional factor *SPL7*, as they are found in its promoter (Figure 1). *SPL7* expression cycles under continuous conditions and this oscillation is almost lost in the *phyB* mutant.<sup>16</sup>

The presence of GTAC boxes, the *cis*-element recognized by the *SPL7* transcription factor,<sup>8</sup> does not correlate with gene induction under Cu deficiency.<sup>13</sup> In addition to positional effects in promoter occupancy and other overlapping *cis*-regulatory elements, temporal factors could also affect dynamic changes in gene expression. In order to understand how such different spatiotemporal phenomena become integrated in the regulation of high-affinity Cu-uptake, a model was proposed for expression activation in response to Cu deficiency, when basal expression of both activator (*i.e.* *SPL7*) and target genes (*e.g.* *COPT2*) is subjected to circadian regulation.<sup>7</sup> A high number of *SPL7*-dependent Cu-deficiency responsive genes<sup>13</sup> show cyclic expression<sup>16</sup>, peaking around 22 h with an expression phase only slightly behind the *SPL7* oscillation (Figure 1). Oscillatory synchronization with the *SPL7* activator appears to be one of the requirements for robust *COPT2* regulation under Cu deficiency.

We propose that a temporal phase synchrony for rhythmic Cu responses leads to a “*constructive interference*”, in analogy to the physical concept for wave interactions.<sup>7</sup> In Cu sufficient media, *COPT2* displays a basal level of periodic expression probably mediated by light and circadian clock rhythms. In response to Cu deficiency, *COPT2* expression is not only induced (around 10 times) by *SPL7*-dependent transcriptional activation, but the amplitude of the oscillation also increases 10 fold without changes in phase or period. Thus, Cu deficiency responses of genes with a basal cyclic expression pattern, such as *COPT2*, are modulated according to their phase timing, such that they are

more potentiated the more their phase coincide with that of the *SPL7* activator<sup>15</sup> (Figure 1). Since *COPT2* expression is 10-fold higher at its maximum than at its minimum, the Cu influx rate ( $J_{10 \times COPT2} / J_{1 \times COPT2}$ ) would be also 10-fold higher around dawn than at dusk, assuming a concomitant effect on COPT2 membrane transporter density and unaltered external Cu concentration.

#### **Copper content regulates *SPL7* function and affects the expression of circadian clock components**

In addition to Cu control, light and circadian regulation, COPT transporters affect, in turn, the function and expression of their own regulators by means of the substrate (Cu) they transport. In this sense, Cu sufficiency inhibiting *SPL7* function<sup>8</sup> forms a negative autoregulatory feedback loop that has been proposed to produce an oscillation in *COPT2* expression, and subsequently, in intracellular Cu levels with an approximate circadian period<sup>20</sup>.

Cu content has been shown to affect the expression of some central components of the circadian clock,<sup>15, 21</sup> such as the single Myb domain transcription factors *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) (for review,<sup>2</sup>) and the protein *GIGANTEA* (*GI*), known to modulate other circadian clock components (for review,<sup>22</sup>). *LHY* and *GI* include, respectively, three and two GTAC boxes in their promoter regions, respectively, which suggests a direct involvement of *SPL7* in their increased abundance under Cu deficiency (Figure 1). In addition to a phase difference with the oscillating *SPL7* expression, multiple reciprocal and indirect effects that *GI* may have on other circadian clock and light signaling components, complicates a straightforward prediction of the effects of Cu on the expression of various signaling factors.

Disturbance from Cu homeostasis affects circadian-clock outputs, which suggests the existence of a reciprocal influence between Cu transport and circadian rhythms.<sup>15, 21</sup> Light signaling and Cu homeostasis pathways are coordinated through the interaction of the transcription factors *SPL7*<sup>15</sup> and *ELONGATED HYPOCOTYL5* (*HY5*).<sup>23</sup> This mutual dependence of Cu transport, circadian clock, and light signaling pathways would lead to the synchronization of inextricably linked outputs from these intertwined and cyclic processes. Moreover, this could explain adaptive advantages based on both increased clock robustness and an optimal scheduling of other cellular cycles according to their Cu dependence.<sup>20</sup>

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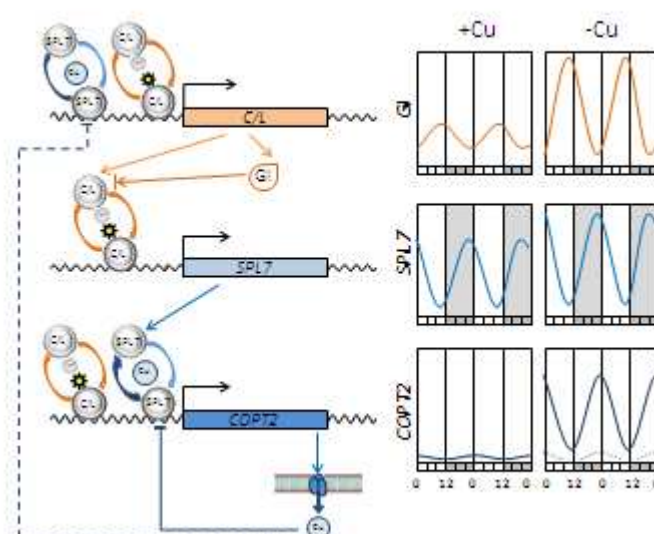
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**Figure 1** Model illustrating the integration of circadian clock and light components in the *SPL7*-regulated Cu deficiency responses.

The promoters of the Cu homeostasis transcription factor *SPL7* and its target, the Cu transporter *COPT2*, contain conserved elements for circadian clock and light signaling (C/L) regulation. Their basal expressions are subjected to circadian and light regulation with almost coincidental phases (22 h for *SPL7* and 0 h for *COPT2*). Involvement of light on *COPT2* expression is evidenced by a drastic reduction of the oscillations amplitude as observed under continuous darkness (discontinuous lines in *COPT2* panel). When the regulator (*SPL7*) is activated by an input (Cu deficiency), synchronous oscillations lead to *constructive interference* and thereby potentiating target gene expression (*COPT2*). Intracellular Cu levels, modulated by *COPT2* transporter activity, not only regulate *SPL7* function and therefore expression of its own transporter, but also affect the expression of circadian clock elements, such as *GI*, which is down-regulated by Cu. Oscillating luciferase activity driven by promoters regions of *GI* and *COPT2* in *pGI:LUC* and *pCOPT2:LUC* transgenic plants, respectively, is shown under constant light conditions and after 12 h light/12 h dark cycle entrainment (subjective night period is shaded grey on the X axis). *SPL7* expression, obtained by RT-PCR quantification, is shown from WT plants grown in light/dark cycles (night period is indicated in grey bars). Cu modulations are shown in blue and circadian/light influences in orange. Positive and negative effects are indicated as pointed and blunt arrows, respectively.