Title: Interaction Between ABA Signalling and Copper Homeostasis In *Arabidopsis thaliana*

Running title: Copper and ABA Homeostasis Interplay

Corresponding author: Dr. L. Peñarrubia, Departamento de Bioquímica y Biología Molecular. Facultad de Ciencias Biológicas. Universitat de València. Avda. Dr. Moliner, 50. 46100-Burjassot, Valencia, Spain. E-mail: lola.penarrubia@uv.es

Subject areas: (2) environmental and stress responses, (7) membrane and transport.

Black and white figures: 6

Colour figures: 2

Supplementary material: 11 Figures and 1 Table.

Accepted for publication in **Plant and Cell Physiology** (April 25 2016)

https://academic.oup.com/pcp/article/57/7/1568/2755867

https://doi.org/10.1093/pcp/pcw087

Title: Interaction Between ABA Signalling and Copper Homeostasis In *Arabidopsis thaliana*

Running title: Copper and ABA Homeostasis Interplay

Authors: Àngela Carrió-Seguí[1†], Paco Romero[1#†], Amparo Sanz[2] and Lola Peñarrubia[1].

Authors addresses:

[1]Departamento de Bioquímica y Biología Molecular. Universitat de València. 46100-Burjassot, Spain.

[2]Departamento de Biología Vegetal. Universitat de València. 46100-Burjassot, Spain.

[#]Present address: Section of Plant Biology, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, United States.

[†]Both authors contributed equally to this work.

SUMMARY

Abscisic acid (ABA) is involved in plant responses to non-optimal environmental conditions, including nutrient availability. Since copper (Cu) is a micronutrient of primary importance, uncovering how ABA affects Cu uptake and distribution is relevant to ensure adequate Cu nutrition in the plants subjected to stress conditions. Inversely, knowledge on how the plant nutritional status may interfere with ABA biosynthesis and signalling mechanisms is necessary to optimise stress tolerance in horticultural crops. Here the reciprocal influence between ABA and Cu content was addressed by using knockout mutants and overexpressing transgenic plants of high affinity plasma membrane Cu transporters (*pmCOPT*) with altered Cu uptake. Exogenous ABA inhibited *pmCOPT* expression and drastically modified *COPT2*-driven localisation in roots. ABA regulated *SPL7*, the main transcription factor responsive for Cu-deficiency responses, and subsequently affected its targets expression. Conversely, ABA biosynthesis (*aba2*) and signalling (*hab1-1 abi1-2*) mutants differentially responded to ABA according to Cu levels. Alteration of Cu homeostasis in *pmCOPT* mutants affected ABA biosynthesis, transport and signalling as genes such as *NCED3*, *WRKY40*, *HY5* and *ABI5* were differentially modulated by Cu status and in the *pmCOPT* and ABA mutants. Altered Cu uptake redounded in modified plant sensitivity to salt-mediated increases in endogenous ABA. The overall results provide evidence for a reciprocal crosstalk between Cu status and ABA metabolism and signalling.

Key words: Abscisic acid, *Arabidopsis thaliana*, copper deficiency, COPT, hormonal signalling, nutrient homeostasis.

Introduction

Copper (Cu) is one of the essential mineral elements needed for normal plant growth and development as it plays a key role in electron transport chains and in redox reactions. Cu also functions as an enzymatic regulator and its deficiency leads to a number of metabolic and developmental alterations that affect vegetative and reproductive processes (Marschner and Marschner, 2012; Yruela, 2013). Most soils have sufficient Cu to sustain plant growth, although scarcity occurs frequently as a consequence of high levels of organic matter or of excess nitrogen-phosphorus-potassium fertilisation (Dobermann and Fairhurst 2000). When bioavailable external Cu levels are low, plants use an energy-consuming system, by which Cu^{2+} is reduced to Cu^{+} by plasma membrane NADPH-dependent cupric reductases (Bernal et al., 2012), and is then taken up through high-affinity COPT transporters. As recently reviewed (Puig, 2014; Peñarrubia et al., 2015), three plasma membrane members of this family, *COPT1*, *COPT2* and *COPT6* (*pmCOPT*), are induced in *Arabidopsis* under Cu-deficiency conditions (Sancenón et al., 2003; Garcia-Molina et al., 2013). *COPT1* is expressed mostly in the root apex and pollen, which indicates its participation in Cu^{+} uptake from soil and redistribution to reproductive organs (Sancenón et al., 2004). COPT2 is present in the root elongation zone and responds to Cu and iron (Fe) deficiencies (Perea-García et al., 2013). Since it is the most highly expressed member of this family of transporters, it can be used as a good deficiency marker in Cu-starved plants (Andrés-Colás et al., 2013). COPT6 has been mainly localised at the vasculature of green tissues and reproductive organs, where it can facilitate Cu redistribution (Jung et al., 2012; Garcia-Molina et al., 2013). The up-regulation of these transporters in response to Cu deficiency is mediated by the zinc (Zn) finger transcription factor Squamosa Promoter Binding Protein-Like7 (SPL7) (Yamasaki et al., 2009; Bernal et al., 2012), where displacement of Zn^{2+} by Cu^{2+} from Zn finger domains may constitute the repressor mechanism by which SPL7 is not functional under Cu-sufficiency conditions (Sommer et al., 2010). Consequently, *Arabidopsis spl7* mutant plants exhibit poor responses to Cu starvation (Yamasaki et al., 2009; Bernal et al., 2012; Zhang et al., 2014).

Plant hormones act as major endogenous cues that regulate morphological, physiological and molecular adaptive responses to environmental changes. Variations in root development in accordance with Cu availability have been indicated (Lequeux et al., 2010; Peto et al., 2011; Yuan et al., 2013). Abscisic acid (ABA) is known to play a pivotal role in plant responses to stress conditions, particularly those that induce water stress, such as drought, cold or salinity (Seki et al., 2003). The involvement of increased ABA levels in responses to salinity is well documented (Xiong and Zhu, 2002), and cell tolerance has been related to the capacity of ABA synthesis under

stress (Perales et al. 2005). ABA is also involved in responses to stresses caused by excess and scarce mineral elements in the root environment (Rubio et al., 2009). High Cu concentrations (100 µM) induce increases in ABA in rice plants (Kim et al. 2014). Such an increase can result from enhanced biosynthesis or reduced catabolism. Thus the first step in ABA degradation to phaseic acid has been impaired in rice by 30 µM Cu as *ABA8Ox* expression was repressed (Ye at al. 2014). Deficiencies of nutrients, such as K or Fe, have also been shown to induce changes in the endogenous ABA contents in different plants (Peuke et al. 2002; Lei et al., 2014). Despite very few data being available on the effect of Cu deficiency on phytohormones levels, interactions between plant Cu status and ABA concentration should be expected since Cu is needed for the biosynthesis of the molybdenum (Mo) cofactor (MoCo) (Schwarz and Mendel, 2006).

In addition to biosynthesis and catabolism, Cu levels can also affect ABA signalling. ABA Insensitive 5 (ABI5), a transcription factor of the ABA signalling pathway, participates in the integration of light and ABA signalling since the well-known light signalling transcription factor Elongated Hypocotyl 5 (HY5) binds to the *ABI5* promoter, and ABI5 overexpression restores ABA sensitivity in the *hy5* mutant (Chen et al., 2008; Lau and Deng, 2010). Interestingly, HY5 has been recently shown to interact with SPL7 (Zhang et al., 2014), the master regulator of Cu-deficiency responses. In line with this, other results have indicated that Cu homeostasis could be affected by ABA levels. Thus an ABA-deficient citrus mutant, prone to fruit dehydration, was unable to induce early molecular responses to mild water stress observed in wild-type fruit (Romero et al., 2012). Of the differential transcriptomic responses noted among cultivars, some have been related with Cu homeostasis, such as the expressions of *COPT1*, *COPT2* and *COPT5*, which were induced in parental, but not in the ABA-deficient, citrus fruit (Romero et al., 2012). This result supports the idea that an ABA/drought-mediated regulation of genes being involved in metal homeostasis. Accordingly, an *in silico* search of putative hormone-responsive *cis*-regulatory elements in the promoters of the high affinity COPT transporters in both *Arabidopsis thaliana* and *Oryza sativa* rendered 45 and 79 ABA-responsive elements, respectively, of which ABRE, MYB/MYC and DPBF were the most abundant (Peñarrubia et al., 2015). Presence of drought- and ABA-related motifs in *COPTs* promoters suggests that these transporters can be regulated under developmental or environmental stressful conditions. Therefore, the relationship between ABA and Cu homeostasis in *Arabidopsis* plants was studied herein by determining the differential responses of knockout and overexpressing *pmCOPT* mutants on Cu- and ABA-related genes expression according to different Cu statuses and ABA treatment. Conversely, the *ABA-deficient 2* (*aba2*) mutant (Leon-Kloosterziel et al., 1996) and the double

Hypersensitive to ABA1 and ABA-Insensitive1 (*hab1-1 abi1-2*) mutant, which encodes protein phosphatases type 2C that act as negative regulators of the ABA signalling pathway (Koornneef et al., 1984; Saez et al., 2004), have been used to understand the effect of ABA on Cu homeostasis-related gene expression.

Results

Sensitivity to Exogenous ABA is Enhanced in Knockout but Reduced in Overexpressing pmCOPT Mutants

To check the influence that Cu homeostasis might have on ABA responses in *Arabidopsis* seedlings, the sensitivity to the hormone of the different mutants with an altered expression in *pmCOPT* transporters was analysed by treatments that employed different ABA and Cu concentrations. Addition of the specific Cu chelator bathocuproinedisulphonic acid disodium (100 µM BCS) was required to obtain severe Cu deficiency conditions since standard commercial ½ MS medium just falls within the mild Cu deficiency range (Andrés-Colás et al., 2013). To generate Cu sufficiency, 1 µM Cu was added to the ½ MS medium, whereas 25 µM were added for Cu excess. As the ABA concentration increased, root length shortened for all the genotypes and Cu conditions (Fig. 1A). Globally, ABA sensitivity reduced as Cu decreased in the medium. Thus under Cu excess (25 µM), root growth was almost completely hindered by 0.6 µM ABA (Fig. 1A) and the effect diminished with lower Cu concentrations, as indicated by the slopes of the regression lines obtained for each genotype (inset in Fig. 1A). It is noteworthy that with sufficient and excess Cu, *copt2* and the triple *copt1 copt2 copt6* knockout mutants showed greater sensitivity to ABA (higher slope values) than the WT seedlings which, in turn, were more sensitive than the *COPT1*^{OE} mutant. In contrast under Cu deficiency, the slope values lowered and the differences among genotypes became less marked, which indicates a stronger effect of ABA in the presence of Cu, particularly in knockout mutants (Fig. 1A). Since differences between phenotypes were significant at 0.6 µM ABA, this ABA concentration was used in the following experiments. The observed pattern of sensitivity to ABA in root length was further confirmed by the green cotyledons rate (GCR) (Supplementary Fig. S1A). The ABA-treated *copt2* and the triple knockout mutant seedlings showed a lower GCR than the WT, while the overexpressing *COPT1*^{OE} mutant was less sensitive to ABA than the control seedlings under the Cu-sufficiency conditions. These differences were lost under the Cu-deficiency conditions (Supplementary Fig. S1A).

The relationship between Cu availability and ABA metabolism was studied by measuring the endogenous hormone levels in the control and ABA-treated WT seedlings grown at different Cu statuses. The seedlings grown under Cu sufficiency showed a significantly higher ABA content compared to the severe Cu deficiency conditions (Supplementary Fig. S1B). In both cases, exogenous ABA treatment triggered an approximate12-fold increase in hormone accumulation. Consequently, the ABA levels after hormone treatment under Cu deficiency were lower than under Cu-sufficiency (Supplementary Fig. S1B). In this context, it is also interesting to note in the non-ABA-treated plants that the endogenous ABA content in the WT and *COPT1*^{OE} seedlings was higher than in the *copt2* and the triple *copt1 copt2 copt6* knockout mutants under Cu sufficiency, whereas these differences were lost under the Cu-deficiency conditions, or even reversed under Cu excess (Fig. 1B). Similarly to the WT, the ABA levels in the *COPT1*^{OE} seedlings were significantly lower under Cu deficiency than under Cu optimal conditions, whereas no variation in ABA content in the *copt2* mutant was noted between these growth conditions (Fig. 1B). ABA content diminished with Cu excess compared to Cu sufficiency and deficiency in all the genotypes, except in the triple *copt1 copt2 copt6* knockout mutant (Fig. 1B). The effect of exogenous ABA on Cu content was also analysed under the three Cu conditions. No significant differences in Cu content were found among genotypes under the Cu-deficiency conditions, regardless of the exogenous ABA treatment (Fig. 1C). In contrast when ABA was absent, the *copt2* and the triple knockout mutants displayed significantly lower Cu levels for sufficient and excess Cu than the WT and *COPT1*^{OE} seedlings, which displayed similar or increased Cu contents for sufficient and excess Cu, respectively. Interestingly, the exogenous ABA treatment significantly reduced the Cu content of the WT and *COPT1*^{OE} seedlings, but not that of the knockout mutants (Fig. 1C).

Exogenous ABA Inhibits the Expression of pmCOPT and Other Cu Homeostasis-Related Genes

Recently reported data (Peñarrubia et al., 2015) have shown that putative ABA-responsive *cis*-regulatory elements (ABREs) are present in the promoter region of *pmCOPT* transporters. This suggests that the expression of these transporters can be influenced by ABA. To better address the effects that ABA can have on Cu homeostasis, this *in silico* analysis has been extended to other Cu-deficiency induced genes (Yamasaki et al., 2009), such as divalent metal transporter *ZIP2*, Fe-superoxide dismutase *FSD1*, and the main transcription factor that regulates Cu-deficiency responses, *SPL7*, all of which have been found to contain a large number of ABREs elements in their promoters (Supplementary Fig. S2).

The effect of ABA on the molecular mechanisms that modulate Cu homeostasis has been analysed by the transcriptional analysis of those genes in the ABA-treated plants grown according to Cu-deficiency and sufficiency statuses. The *spl7* mutant was included in these analyses to decipher whether the regulation carried out by ABA is dependent on SPL7 (Fig. 2). Exogenous ABA treatment consistently lowered the expression levels of the *pmCOPT* genes in the WT when grown under the Cu-sufficiency conditions (Fig. 2A and Supplementary Fig. S3A). In contrast, when Cu availability decreased, and although *pmCOPTs* were up-regulated, the exogenous ABA treatment did not apparently affect their expression (Fig. 2B and Supplementary Fig. S3B). Taking into account the different scales shown in Figure 2, it becomes evident that the transcript levels of *COPT2* displayed a more drastic increase (15-fold) than the other *pmCOPT* transporters (Supplementary Fig. S3). These inductions almost went undetectable in the *spl7* mutant, which confirms that *COPT2* is the best SPL7-dependent COPT marker for Cu deficiency. Unlike that found for *pmCOPT*, the *SPL7* expression levels were not regulated by Cu availability and did not consequently vary significantly when Cu was present in the growing media (Fig. 2). Surprisingly, *SPL7* was repressed by ABA under Cu-sufficiency conditions, but not with Cu deficiency, which might account for at least part of the reduction noted in its targets expressions. A further reduction in *COPT2* expression still took place in the *spl7* mutant, which suggests that ABA also plays a role independently of the SPL7 function (Fig. 2). The *ZIP2*, *YSL2* and *FSD1* expression patterns were most consistent with those of *pmCOPT* in the WT plants (Fig. 2 and Supplementary Fig. S3). Hence these gene expressions were induced by Cu deficiency in an SPL7-dependent manner, and ABA treatment significantly lowered the transcript levels in the WT seedlings under Cu-sufficiency conditions (Fig. 2 and Supplementary Fig. S3).

The Root Growth Phenotype of ABA Mutants Depends on Cu Status

To uncover the putative role of Cu in ABA-dependent root length, we used two ABA mutants in a root growth assay with different Cu statuses (Fig. 3), the *aba2* mutant, which presents a blockage of the ABA biosynthetic pathway upstream of the *NCED* catalysed step (Schwartz et al. 1997) and hence lowered ABA levels, and the double *hab1-1 abi1-2* mutant, which exhibits hypersensitivity to the hormone (Saez et al., 2006).

Under all the tested Cu conditions, the *aba2* mutant plants were more resistant to root growth inhibition by exogenous ABA than the WT plants, whereas the *hab1-1 abi2* mutants were more sensitive (Fig. 3A) in accordance with their ABA-dependent phenotype. As previously indicated, Cu is required for reducing root growth in all the

tested genotypes since this reduction is impaired by severe Cu deficiency (Fig. 3A). So whereas WT root reduction was around 50% in the presence of Cu, it was not significant with severe deficiency. The *aba2* mutant was mostly resistant to root growth inhibition under any Cu condition, while *hab1-1 abi1-2* mutant hypersensitivity to ABA required Cu (Fig. 3A). Due to this hypersensitivity, it was not possible to determine the Cu content in these plants (Fig. 3B). Although Cu content remained unaffected by severe Cu deficiency, it increased in the ABA mutants with Cu excess in the presence and absence of ABA (Figure 3B). Taken together, these results indicate that Cu status affects ABA responses and is, in turn, influenced by ABA.

The Expression of Cu Homeostasis-Related Genes is Altered in ABA Mutants

To further uncover how ABA affects Cu homeostasis, the expression of Cu-deficiency markers was studied in the ABA biosynthesis and signalling mutants with different Cu statuses (Fig. 4). In general, with low Cu, the expression of Cu-deficiency markers increased in the *aba2* mutant and reverted to the WT levels after treatment with exogenous ABA, as expected for an ABA-deficient mutant (Fig. 4A). Interestingly, *SPL7* expression slightly increased in the *aba2* mutant, but was not modulated by exogenous ABA for any Cu status (Fig. 4), which allowed endogenous/exogenous ABA effects to be differentiated. Thus whereas regulation by endogenous ABA could be, at least in part, mediated by the increase in the transcriptional activator, the effects of exogenous ABA were not dependent on *SPL7* expression levels. With sufficient and excess Cu, the expected increase in the responses of the Cu-deficiency markers in the *aba2* mutant and the reversion by exogenous ABA were lost (Figs. 4B and 4C). Instead, a more or less marked decrease was observed, which also occurred for *SPL7* for Cu sufficiency.

Cu-deficiency markers expressions mostly increased in the *hab1-1 abi1-2* mutant and the effects after ABA treatment varied depending on the gene and Cu conditions (Fig. 4). Once again, *hab1-1 abi1-2* mutant hypersensitivity to ABA in the presence of Cu prevented these samples from being analysed. With Cu deficiency, the effect of exogenous ABA was SPL7-independent since, as indicated above, *SPL7* expression was not affected by ABA treatment, whereas distinct and significant effects were measured for the analysed Cu-deficiency markers (Fig. 4A). With Cu sufficiency and excess, the similar expression levels of the Cu-deficiency markers in the *hab1-1 abi1-2* mutant and WT plants (Figs. 4B and 4C) indicate that endogenous ABA signalling slightly modulates Cu responses. Taken together, these data indicate a complex scenario where Cu status could affect ABA homeostasis in multiple steps, such as biosynthesis and signalling and, vice versa, ABA alterations could drive changes in Cu homeostasis.

ABA and Cu Status both Influence Oxidative Stress Levels

To uncover whether ABA treatment influences oxidative stress status according to Cu availability, we studied plasma membrane-associated NADPH oxidase RBOHD, which has been reported to be involved in ABA-promoted ROS production (Kwak et al., 2003). *RBOHD* gene expression was not dependent on SPL7, but was induced by Cu deficiency in all the studied genotypes. Unlike that found for the SPL7-dependent genes, ABA treatment generally lowered *RBOHD* gene expression under both Cu-sufficiency and Cu-deficiency conditions (Supplementary Fig. S3).

H_2O_2 was detected in the WT, *pmCOPT*, *spl7* and *aba2* mutants subjected to ABA treatment, and was grown under Cu-sufficiency and Cu-deficiency conditions (Supplementary Fig. S4). For Cu sufficiency, H_2O_2 production in the WT was poor and no significant influence of ABA treatment was observed. In contrast, oxidative stress was evident in the roots of the WT seedlings grown under Cu-deficiency conditions, and regardless of ABA treatment (Supplementary Fig. S4). Under Cu sufficiency conditions, however, *copt2* seedlings drastically increased oxidative stress after ABA treatment, whereas ROS accumulation became evident in $COPT1^{OE}$, even without hormonal treatment (Supplementary Fig. S4). It was noteworthy that the *aba2* mutant behaved similarly to the *copt2* seedlings, and that the *spl7* and *copt1 copt2 copt6* mutants also showed increased ROS content after ABA treatment when grown under optimal Cu-availability conditions (Supplementary Fig. S4).

ABA Modifies the Spatial COPT2 Gene Expression Pattern

The effect of ABA on the spatiotemporal *COPT2*-driven expression pattern was analyzed through *pCOPT2::GUS* (Perea-García et al., 2013) and *pCOPT2::LUC* (Perea-García et al., 2016) transgenic plants. Since this gene is expressed mainly under Cu-deficiency conditions, we selected a mild metal deficiency (½ MS) to allow gene induction. In 7-day-old seedlings, *GUS* expression was located mainly at the cotyledon apex, on the basis of true leaves and along root tissues (Fig. 5A-D). The exogenous ABA treatment did not modify the distribution of *COPT2*-driven *GUS* expression in aerial parts, whereas *GUS* expression was almost abolished from tissues along the root, and concentrated in the root crown and root hairs after hormone treatment (Fig. 5E-H). Increasing ABA concentrations did not modify the parameters related with the daily *COPT2*-driven oscillation pattern, such as amplitude, phase and period (Supplementary Fig. S5). Whether lack of ABA affects temporal regulation was further assessed in the *pCOPT2::GUS* plants subjected to ABA treatments at different times (Supplementary Fig. S6). The

results demonstrate that *COPT2*-driven gene expression consistently focused on the root crown and root hairs zones in the ABA-treated seedlings at the same times as the control samples, and induction occurred at the transcript levels along root tissues (Supplementary Fig. S6).

ABA Biosynthesis and Signal Transduction Vary with Cu Availability

To study the effects of Cu availability on hormonal content, WT seedlings were grown within a wide range of Cu concentrations. Compared to Cu sufficiency, both severe Cu deficiency and moderate metal excess displayed lower ABA levels (Fig. 6A). Therefore, optimal Cu availability is required for normal endogenous ABA content in *Arabidopsis* seedlings. No significant differences in auxin (IAA) and jasmonate (JA) contents were detected under the assayed experimental conditions (Supplementary Fig. S7A). Likewise, no significant differences in IAA and JA contents were found for Cu availability and ABA treatment (Supplementary Fig. S7B).

The influence that Cu homeostasis alteration can have on the expression of ABA metabolic and signalling genes was studied by taking advantage of the RNA-Seq analysis performed by Bernal et al. (2012), in which responses to Cu deficiency conditions were compared in root and shoot tissues from WT and *spl7* knockout mutant plants. After analysing the transcriptional response of about 60 of the genes involved in ABA biosynthesis and ABA signalling, those genes that displayed value that changed at least 2-fold compared with the Cu sufficiency conditions are shown (Figs. 6B and 6D, respectively). Induction by Cu deficiency prevailed among the genes related to ABA metabolism in the shoots of WT plants, and in the shoots and roots of *spl7* mutants (Figs. 6B and 6D). This analysis allowed us to address the influence of the SPL7 transcription factor on the regulation of these ABA-related genes. Complex differential SPL7 responsiveness of ABA biosynthetic genes was envisaged and these responses were also organ-dependent (Fig. 6B). Several genes showed the same expression pattern in WT and *spl7* mutants, which indicates SPL7-independent Cu regulation. This was the case of *NCED2*, *NCED3*, *NCED5* and *UGT71B6* in shoots. However, an SPL7-dependent gene expression pattern was obtained for *NCED2*, *NCED4*, *NCED5*, *CYP707A1* and *UGT71B6* in roots (Fig. 5B). In this context, it is important to note that *NCED3* has been shown as the most expressed gene to codify for the limiting step in ABA biosynthesis positive feedback regulation (Barrero et al., 2006). For this reason, we checked the expression pattern of *NCED3* at different Cu concentrations (Fig. 6C). Strikingly, the *NCED3* transcript levels described an opposite pattern to that of ABA content under the same experimental conditions (Figs. 6A and 6B), and with higher expression levels under both severe Cu deficiency and

excess Cu conditions compared to the Cu-sufficiency growing conditions. These results demonstrate that Cu availability affects ABA biosynthesis. The *in silico* analysis revealed that ABA signalling genes were also affected by Cu availability (Fig. 6D). Nevertheless, of the more than 30 ABA signalling-related genes evaluated, only a couple of PP2CA and seven transcription factors were differentially affected by Cu status, and attained the 2-fold threshold (Fig. 6D). Most of these signalling genes were differently regulated in the WT and *spl7* mutant seedlings in root and shoot tissues, which suggests SPL7-dependent regulation (Fig. 6D). In line with this, we found that the promoter regions of some of these ABA-related genes contained putative Cu-responsive *cis*-regulatory elements (CuREs) (Supplementary Fig. S2), which indicates reciprocal regulation between Cu homeostasis and ABA metabolism and signalling.

To gain a better insight into this mutual regulation, we studied the effect of ABA and Cu availability on the transcriptional regulation of *NCED3, HY5, WRKY40* and *ABI5*. Based on the differential sensitivity to ABA observed in the *copt2* knockout and *COPT1*^{OE} overexpressing mutants (Fig. 1), we included these genotypes in our assays (Fig. 7). In general, the expression of the *NCED3* gene was induced by ABA treatment under Cu sufficiency in all the tested genotypes (Fig. 7A). Although the basal expression level of *NCED3* under the Cu-deficient conditions was higher than that under the Cu sufficiency conditions in WT seedlings, the fold changes in the transcript levels of this gene after ABA treatment significantly lowered for Cu deficiency compared to Cu sufficiency (4.5 *vs.* 1.9 fold; Fig. 7). Moreover, the expression levels of ABA biosynthetic gene *NCED3* were higher in the *pmCOPT* mutants than in the WT when ABA was present. Despite *NCED3* induction by Cu deficiency in the WT seedlings (Figs. 6B-C and 7), in the genotypes that showed altered Cu homeostasis (*spl7*, *copt2* and *COPT1*^{OE}), *NCED3* expression was drastically decreased (Fig. 7). Under optimal Cu conditions, the *HY5* and *WRKY40* expression levels were generally reduced by ABA, although *WRKY40* expression was also induced by Cu deficiency, and the *HY5* transcript levels increased in response to ABA treatment with severe Cu deficiency (Fig. 7). The expression levels of the ABA signalling gene, *WRKY40*, did not significantly change in the *pmCOPT* mutants in the absence and presence of ABA. Strikingly, certain genes showed an increased expression in specific genotypes, such as *HY5* in *copt2* and *WRKY40* in the *spl7* seedlings when grown under Cu sufficiency (Fig. 7A). A clear trend was observed for the *HY5*, *WRKY40* and *ABI5* transcripts in the non-ABA-treated seedlings for Cu deficiency. Whereas the transcript levels in the *COPT1*^{OE} seedlings were consistently higher than in the WT, their expression levels lowered in the *copt2* mutant

(Fig. 7A). Accordingly, these expression patterns of the ABA signalling genes in the *pmCOPT* mutants matched their observed ABA responses shown in Figure 1.

The same set of genes was also analysed in the *aba2* and *hab1-1 abi1-2* mutants (Supplementary Fig. S8). Their expressions generally increased as Cu decreased in the media. As regards to *NCED3*, the expression in the *hab1-1 abi1-2* mutant was higher than in the WT, independently of the Cu levels (Supplementary Fig. S8). However, the *aba2* mutant showed identical levels than the WT for deficient and excess Cu, but higher levels for Cu sufficiency (Supplementary Fig. S8), which indicates an influence of Cu in the feedback regulatory loop of ABA on its own synthesis. Once again, the *HY5* levels were more affected in the mutants with Cu deficiency, as was *WRKY40* in the *aba2* mutant. Finally, whereas signalling transcription factor *ABI5* was not affected by Cu sufficiency compared to the WT, its levels were regulated in the ABA mutants under the Cu deficiency and excess conditions, which once again suggests a complex interaction of Cu and ABA biosynthesis and signalling (Supplementary Fig. S8).

Sensitivity to Salt Stress is Enhanced in copt2, but Reduced in COPT1^{OE} Mutants

To study whether ABA sensitivity is similarly regulated by Cu availability independently of the ABA signal source (exogenous application/stress-induced endogenous biosynthesis), the WT, *copt2*, *copt1 copt2 copt6* and *COPT1^{OE}* seedlings were subjected to salt stress under both Cu-optimal and Cu-severe deficiency conditions.

Salt stress increased ABA content in all the tested genotypes and under both the Cu-sufficiency and Cu-severe deficiency conditions (Fig. 8A and Supplementary Fig. S9A, respectively). Interestingly, this increment was more marked in the WT than in the *copt2* mutant seedlings under the Cu-sufficiency conditions (Fig. 8A). NaCl treatment barely reduced Cu content in all the genotypes. However, the *copt2* knockout mutant seemed to be affected more by salt stress, and had a significantly lower metal content than the *COPT1^{OE}* seedlings (Fig. 8B). In contrast under Cu deficiency, no differences in either Cu content or ABA levels were found among the genotypes, regardless of being under the control conditions or subjected to salt stress (Supplementary Fig. S9A-B).

The response to stress-induced endogenous ABA variations was studied by determining root length and changes in chlorophyll content index (CCI) after salt stress since both are classical parameters used to describe the effect of ABA and the severity of applied salt stress. As with the exogenous ABA treatment (Fig. 1A), the root lengths of the *copt2* and *copt1 copt2 copt6* knockout mutants were affected more by stress than the WT which, in

turn, had a lower root length ratio than the *COPT1*^{OE} seedlings under Cu-sufficiency conditions (Fig. 8C). This pattern was confirmed by studying the changes in CCI. As shown in Fig. 8D, the *copt2* and triple knockout seedlings were more sensitive to stress than the WT, while the overexpressing *COPT1*^{OE} mutant showed a higher CCI. The differences among the genotypes in CCI were lost under the Cu-deficiency conditions, whereas the root length ratio indicated that the *copt2* and the triple knockout mutants were more resistant to salt stress when Cu was scarce (Supplementary Fig. S9C-D). These results indicate that knockout mutants are more sensitive to ABA variations since stress-induced endogenous ABA was lower (Fig. 8A), while the reductions in Cu content, root length ratio and CCI were higher than in the WT and the overexpressing mutant (Fig. 8B-D).

In line with this idea, the transcriptional regulation of Cu-related homeostasis genes by salt stress-mediated ABA increments under Cu optimal conditions mostly paralleled that obtained with the exogenous ABA treatment, except for *COPT1* in the WT seedlings. Thus NaCl treatment significantly lowered the *pmCOPT* and *SPL7* gene expression levels in the 14-day-old WT, *copt2* and *COPT1*^{OE} seedlings (Supplementary Fig. S10A). Under these experimental conditions, we also studied the effect of Cu availability on the transcriptional regulation of the ABA-biosynthetic (*NCED3*) and signalling (*HY5*, *WRKY40* and *ABI5*) genes. *NCED3* was induced by Cu deficiency, and also by salt stress, but only under Cu optimal conditions, and not in *pmCOPT* mutants (Supplementary Fig. S11). Similarly, *pmCOPT* mutants did not achieve the *ABI5* expression levels observed in the WT, regardless of the growing media or stress applied. *HY5* and *WRKY40* responses were also affected by Cu status and in *pmCOPT* mutants. Whereas the *HY5* transcript levels slightly decreased by Cu deficiency and were repressed by salt stress, the *WRKY40* gene expression was induced by both Cu deficiency and endogenous increases in the ABA levels caused by abiotic stress under the Cu-optimal growing conditions (Supplementary Fig. S11). Taken together, these results indicate that both ABA biosynthesis and signalling produced by environmental stress conditions, such as high salt, are affected by Cu status.

Discussion

Understanding molecular responses of crop plants to the abiotic stresses in which ABA is involved is critical for facing environmental challenges in which the crosstalk between ABA and nutrient homeostasis plays a central role (Boursiac et al., 2013; Peñarrubia et al., 2015). In the present work, the crosstalk between Cu homeostasis and ABA responses is explored. Attenuated ABA responses were observed under Cu deficiency (Figs.

1A, 1B, 3A and Supplementary Fig. 1). This indicates that ABA effects depend on plant Cu status, which could be evidenced by either modifying the Cu content in the medium or studying transgenic plants affected in Cu transport. ABA affects Cu-deficiency markers expression in a complex process that depends on Cu status (Figs. 2, 4 and 5).

Although increased stress ABA levels have been described for higher (100 μM) Cu concentrations (Kim et al., 2014; Ye et al., 2014), no increases in stress ABA content were detected (Fig. 6A) within the range of the mild Cu concentrations used herein (0-25 μM). Instead a slight decrease in ABA content was observed in Cu deficiency and excess compared to Cu sufficiency (Fig.6A). The Cu requirement for normal ABA content can be explained by the role that Cu plays on the molybdenum cofactor (MoCo) biosynthesis pathway (Schwarz & Mendel 2006). The effect of Cu excess can be due to the fact that Cu may compete with Mo during MoCo biosynthesis (Kuper et al., 2004). Thus Mo content increased under the Cu deficiency conditions (Supplementary Fig. S7C), as also reported in *Brassica napus* under Cu deficiency (Billard et al., 2014). Although Cu effects have been proposed for other hormones (Wu et al., 2012; Yan and Dong, 2014; Arteca and Arteca, 2007; Maksymiec, 2007; Kazan, 2013; Lequeux et al. 2010; Peñarrubia et al., 2015), no significant changes were observed for auxins and jasmonates at the mild Cu levels used herein (Supplementary Fig. S7).

ABA biosynthesis genes, such as *NCED3* expression, were increased by exogenous ABA (Fig. 7). The fact that this induction was repressed in BCS and in Cu^+ transport-altered mutants, *slp7* and *copt2* (Fig. 7), suggests a key effect of spatiotemporal-regulated Cu homeostasis on ABA biosynthesis. This regulation is at least partially dependent on SPL7, and the fact that the *NCED3* promoter displays seven putative *GTAC* boxes (Supplementary Fig. S2) agrees with this. Taken together, these results indicate a delicate balance in the negative feedback loop established between Cu requirement for ABA biosynthesis and the regulatory role of this hormone in Cu uptake. Apart from affecting ABA biosynthesis, Cu deficiency drastically modified ABA signalling (Fig. 6D and Fig. 7). The induction of *ABI5* expression by exogenous ABA was greatly compromised under Cu deficiency, according to the low ABA sensitivity exhibited in BCS (Fig. 1A). In agreement with their putative roles as *ABI5* repressors, the expressions of two transcription factors that bind the *ABI5* promoter, WRKY40 and HY5 (Chen et al., 2010; Chen et al., 2014), were down-regulated by ABA in the WT plants under Cu sufficiency (Fig. 7A). It is noteworthy that *HY5* was highly expressed in the *aba2* mutant (Supplementary Fig. S8) and the $COPT1^{OE}$ line under the Cu deficiency conditions, and also in the *copt2* mutant with Cu sufficiency (Fig. 7). These results indicate that both Cu and ABA could well be needed to synergistically repress *HY5*, which would be a plausible explanation for the ABA-related

phenotypes shown by the *copt2* and *COPT1*^{OE} lines (Fig. 1A and Fig. 1B). Since *hy5* mutants are less sensitive to ABA (Chen et al., 2014), consequently the *copt2* mutant, which expressed higher *HY5* levels, gave an enhanced response to ABA under Cu sufficiency (Fig. 1A and Fig. 1B). Since HY5 interacts with SPL7 (Zhang et al., 2014), these results show HY5 as a putative integrator between ABA and Cu deficiency responses.

The attenuation of Cu deficiency responses by exogenous ABA, including the *pmCOPT*s and its transcriptional regulator, SPL7 (Fig. 2), could explain how ABA may affect endogenous Cu content (Fig. 1E). The promoters of *SPL7* and its *pmCOPTs* targets display putative *ABRE cis*-elements (Nakashima et al., 2006; Peñarrubia et al., 2015; Supplementary Fig. S2), which could account for their ABA-mediated negative regulation with Cu sufficiency (Fig. 2A). Thus ABA acts on the target expression both dependently and independently of the SPL7 transcription factor. Other metal transport systems, like Fe and Zn, have also been reported to be affected by exogenous ABA (Lei et al., 2014; Shi et al., 2015), which indicates a widespread effect of ABA on metal transport.

Cu deficiency-related regulation could be explained by interferences in *pmCOPTs* promoters of different transcription factors. Thus the promoters of *pmCOPTs* present both *ABRE* and *CuRE* elements (Supplementary Fig. S2), which could account for ABA and Cu deficiency regulation, respectively (Nakashima et al., 2006; Yamasaki et al., 2009). In the presence of Cu, SPL7 binding to its targets promoters is considerably reduced (Yamasaki et al., 2009). In these circumstances, other ABA-repression factors could bind Cu deficiency targets with ABA-related *cis* regulatory elements in their promoters, such as *pmCOPTs*. According to this suggestion, a slight regulation in *pmCOPTs* expression by ABA was observed in the *spl7* mutant in both Cu regimes (Fig. 2 and Supplementary Fig. S3). Exogenous ABA drastically modified the *COPT2*-driven localization pattern from tissues along the root towards the crown (Figs. 5 and Supplementary Fig. S6). ABA levels in root vascular bundles have been reported to increase under both nutrient shortage (Vysotskaya et al., 2008) and different abiotic stresses, such as drought and salinity (Nambara and Marion-Poll, 2005; Waadt et al., 2014). According to our results, Cu uptake under stress conditions would be inhibited in the locations where increased ABA caused by stress was present, and could be restricted to the locations where ABA remained unchanged. A relevant issue is to determine the importance of *pmCOPTs* expression in the relative resistance of plants subjected to environmental stresses, such as salinity, by inducing increases in ABA. Under our experimental conditions, the answer was that absence of *pmCOPTs* expression under Cu sufficiency led to increased sensitivity (Fig. 8), which mostly reproduced the changes observed after exogenous ABA application (Fig. 1).

Finally, we recently reviewed the relevance of the dynamic adaptation of nutrient availability to growth requirements under environmental constraints, while preventing their toxicity in the case of metal nutrients (Peñarrubia et al., 2015). In this context, hormones probably play a fundamental role in the spatial arrangement and temporal schedule of metal transport processes by affecting plant development. Conversely, metal availabilities could interfere at many levels with hormone biosynthesis transport and signalling processes. These interactions could coordinate and integrate spatio-temporal information to optimise plant metabolism and physiology (Sánchez et al., 2011; Haydon et al., 2013; Peñarrubia et al., 2015), and the understanding of these complex mechanisms is a pressing challenge to face foreseen unfavourable effects of climate change on agriculture.

Materials and Methods

Plant Material and Growth Conditions

Wild-type (WT) plants of *Arabidopsis thaliana*, ecotype Columbia-0 (Col-0), were used in this study in addition to the *copt2*, *copt1 copt2 copt6*, *spl7*, *aba2* and *hab1-1 abi1-2* knockout mutants, the overexpressing $COPT1^{OE}$ mutant, and the *pCOPT2::GUS* and *pCOPT2::LUC* transgenic lines, which have been previously described in Perea-García (2013), Gayomba et al. (2013), Yamasaki et al. (2009), Saez et al. (2006) and Andrés-Colás et al. (2010). Seeds were stratified for 2 d at 4ºC and surface-sterilised with sequential washes in 70% ethanol (5 min), bleach (5 min) and water (2 x 2 min). They were then sown on plates that contained ½ MS (Murashige and Skoog) medium supplemented with 1% sucrose (w/v) and 0.8% agar at pH 5.7. To generate Cu sufficiency, 1 µM $CuSO_4$ was added to the medium, 25 µM $CuSO_4$ for Cu excess and 100 µM BCS (bathocuproinedisulphonic acid disodium) for severe Cu deficiency. Unless otherwise stated, 0.6 µM ABA was selected for hormonal treatments. In the salt stress assays, the 7-day-old seedlings grown under Cu deficiency or sufficiency were transferred to new plates that contained 200 mM NaCl for another 7-day period. Plants were grown under intermediate photoperiodic conditions (12 h light, 20-23°C/12 h darkness, 16°C; LDHC).

Biochemical Determinations

Fresh *Arabidopsis* material was washed once with 20 µM EDTA and 3 times with MilliQ water. For the ABA, IAA and JA determinations, plant material was lyophilised and then analysed by HPLC at the IBMCP

(Valencia, Spain). Cu and Mo contents were determined by ICP-MS at the 'Servicios Centrales de Investigación' (Universidad de Almeria, Spain) after acid digestion (65% (v/v) HNO_3, 80-90ºC) of dried plant material (65ºC, 2 d).

Determination of Physiological Parameters

For primary root length determinations, plates were placed vertically in a growth chamber and roots were measured with the Image J software (http://rsb.info.nih.gov./ij). The green cotyledons rate (GCR) was calculated as the percentage of green cotyledons over total germinated seeds. Leaf chlorophyll was measured as chlorophyll content index (CCI) by a portable chlorophyll meter (CCM-200, Opti Sciences, USA). Average values per plant were obtained from three different leaves. Five plants were analysed for each sampling condition.

RNA Extraction and Transcriptional Analyses

Total RNA was extracted and treated to remove possible genomic DNA contamination, as described in Andrés-Colás et al. (2006). Thereafter, the amount of RNA was measured by a spectrophotometric analysis and its quality was verified by agarose gel electrophoresis and ethidium bromide staining. Reverse transcription, followed by a quantitative PCR analysis (qRT-PCR), was performed and designed according to Romero et al. (2012). Gene-specific primers were designed with the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0). Both synthesised cDNA and primer pairs were thereafter incubated with Platinum SYBR-Green qPCR Super-Mix-UDG with ROX (Invitrogen) at 95ºC for 10 min, followed by 40 cycles of 95ºC for 10 s, 60ºC for 5 s, and 72ºC for 10 s. The forward (F) and reverse (R) sequences for specific primers are shown in Table S1. To transform fluorescent intensity measurements into relative mRNA levels, a 2-fold dilution series of a mixture that contained an equal amount of each cDNA sample was used and standard curves were constructed for all studied genes. The *UBIQUITIN10* reference gene was used for data normalisation. Each sample was analysed in triplicate and the mean ratios ± SE were calculated.

In Silico *Analyses*

The genomic sequences of *Arabidopsis thaliana* were obtained from the Phytozome Database (www.phytozome.net). The promoter sequences were defined as the 2,000 bp upstream of the five prime untranslated region (5'-UTR) from the corresponding genes. Then they were analysed in the PLACE database

(www.dna.affrc.go.jp/PLACE). The Cu-responsive elements (CuRE, GTAC) and the commonest and most abundant ABA responsive elements, including ABRE (ACGTGG/TC), MYB (CNGTTR) and MYC (CANNTG), were identified.

GUS and Luciferase Assays

Assays were performed as described by Jefferson et al. (1987). To perform the bioluminescence assays, the *pCOPT2::LUC* plants were grown for 6 d in an intermediate photoperiod (LDHC) in ½ MS dishes, as described above. Afterwards, seedlings were transferred to a 96-well white plate (Thermo-fisher) with ½ MS medium that contained 3% sucrose, and were supplemented with increasing concentrations of ABA. For the enzymatic reaction, substrate D-luciferine (Sigma) (0.42 mg/mL), resuspended in 0.01% Triton X-100, and Tris-acetate 1 M pH 7.75 buffer were added to each individual well. The plate was closed with optic film cover Microseal B# Film (Biorad). After one more cycle under LDHC conditions (acclimation), the plate was transferred to continuous light and constant temperature conditions (LLHH). Bioluminescence intensity was measured in a Luminoskan Ascent luminometer. Data were processed with the Ascent 2.6 software (Thermo Scientific) and expressed in 'relative light units'.

Oxidative Stress Staining

In order to detect the *in situ* production of hydrogen peroxide (H_2O_2), 3, 3'-diaminobenzidine (DAB) staining was carried out according to Thordal-Christensen et al. (1997) with minor modifications. Seven-day-old plants were grown as described above, incubated in 1 mg/mL DAB solution (pH 3.5) for 4 h in the dark at room temperature and then transferred to 90% (v/v) ethanol at 90ºC to completely remove chlorophylls from green tissues. Prior to imaging, samples were suspended in 10% lactic acid.

Statistical Analyses

The statistical analyses of the relative expression assays (pair-wise fixed reallocation randomisation tests; $P \leq 0.05$) were performed by comparing the expression levels of each particular gene (RT-PCR) by the Relative Expression Software Tool (REST: http://rest.gene-quantification.info; Pfaffl et al., 2001). Chi-squared tests, followed by two-tailed z-tests, were run for the analysis of rates ($P \leq 0.05$). For the other parameters, one-way ANOVAs were

performed. Significant differences between means were established by *post hoc* tests (Tukey or Games-Howell, according to data homoscedasticity; $P \leq 0.05$) using the IBM SPSS Statistics software, version 19.0.0. Data are provided as the mean values ± SD of at least three independent experiments.

Funding

This work was supported by the Spanish Ministry of Economy and Competitiveness and the FEDER funds from the European Union [grants BIO2011-24848 and BIO2014-56298-P (L.P. and A.S.), a Junior Postdoctoral Position within the framework of this Project (P.R.) and a predoctoral FPI fellowship (A.C-S.)].

Acknowledgements

We thank Dr. P. L. Rodríguez (IBMCP-UPV-CSIC) for providing the *aba2* and *hab1-1 abi1-2* mutant seeds and Dr. Teresa Lafuente (IATA-CSIC) for critically reading the manuscript.

Disclosures

The authors have no conflict of interests to declare.

References

Andrés-Colás, N., Perea-García, A., Puig, S and Peñarrubia, L. (2010) Deregulated copper transport affects *Arabidopsis* development especially in the absence of environmental cycles. Plant Physiol. 153: 170-184.

Andrés-Colás, N., Sancenón, V., Rodríguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker, J.R., et al. (2006) The *Arabidopsis* heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J. 45: 225-236.

Andrés-Colás, N., Perea-García, A., Mayo de Andrés, S., Garcia-Molina, A., Dorcey, E., Rodríguez-Navarro, S.,et al. (2013). Comparison of global responses to mild deficiency and excess copper levels in *Arabidopsis* seedlings. Metallomics. 5(9):1234-46.

Arteca, R.N. and Arteca, J.M. (2007) Heavy-metal-induced ethylene production in *Arabidopsis thaliana*. J. Plant Physiol. 164: 1480-1488.

Barrero, J.M., Rodríguez, P.L., Quesada, V., Piqueras, P., Ponce, M.R. and Micol, J.L. (2006) Both abscisic acid (ABA)-dependent and ABA-independent pathways govern the induction of NCED3, AAO3 and ABA1 in response to salt stress. Plant Cell Environ. 29: 2000-2008.

Bernal, M., Casero, D., Singh, V., Wilson, G.T., Grande, A., Yang, H., et al. (2012) Transcriptome sequencing identifies SPL7-regulated copper acquisition genes *FRO4/FRO5* and the copper dependence of iron homeostasis in *Arabidopsis*. Plant Cell 24: 738-761.

Billard, V., Ourry, A., Maillard, A., Garnica, M., Coquet, L., Jouenne, T., et al. (2014) Copper-deficiency in *Brassica napus* induces copper remobilization, molybdenum accumulation and modification of the expression of chloroplastic proteins. PLoS ONE 9: e109889.

Boursiac, Y., Léran, S., Corratgé-Faillie, C., Gojon, A., Krouk, G. and Lacombe, B. (2013) ABA transport and transporters. Trends Plant Sci. 18: 325-333.

Chen, C., Twito, S. and Miller, G. (2014) New cross talk between ROS, ABA and auxin controlling seed maturation and germination unraveled in APX6 deficient Arabidopsis seeds. Plant Signal. Behav. 9: e976489.

Chen, H., Lai, Z., Shi, J., Xiao, Y., Chen, Z. and Xu, X. (2010) Roles of arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. BMC Plant Biol. 10: 281.

Chen, H., Zhang, J., Neff, M.M., Hong, S.W., Zhang, H., Deng, X.W., et al. (2008) Integration of light and abscisic acid signaling during seed germination and early seedling development. Proc. Nat. Acad. Sci. USA 105: 4495-4500.

Dobermann, A. and Fairhurst, T.H. (2000) Rice. Nutrient disorders and nutrient management. Ed. Potash & Phosphate Institute (PPI) and International Rice Research Institute (IRRI).

Garcia-Molina, A., Andrés-Colás, N., Perea-García, A., Neumann, U., Dodani, S.C., Huijser, P., et al. (2013) The *Arabidopsis* COPT6 transport protein functions in copper distribution under copper-deficient conditions. Plant Cell Physiol. 54: 1378-1390.

Gayomba, S.R., Jung, H.I., Yan, J., Danku, J., Rutzke, M.A., Bernal, M., et al. (2013) The CTR/COPT-dependent copper uptake and SPL7-dependent copper deficiency responses are required for basal cadmium tolerance in *A. thaliana*. Metallomics 5: 1262-1275.

Haydon, M.J., Hearn, T.J., Bell, L.J., Hannah, M.A. and Webb, A.A.R. (2013) Metabolic regulation of circadian clocks. Sem. Cell and Develop. Biol. 24: 414-421.

Jung, H.I., Gayomba, S.R., Rutzke, M.A., Craft, E., Kochian, L.V., Vatamaniuk, O.K, (2012) COPT6 is a plasma membrane transporter that functions in copper homeostasis in *Arabidopsis* and is a novel target of *SQUAMOSA* promoter-binding protein-like 7. J. Biol. Chem. 287: 33252-33267.

Kazan, K. (2013) Auxin and the integration of environmental signals into plant root development. Ann. Bot. 112: 1655-1665.

Kim, Y.H., Khan, A., Kim, D.H., Lee, S.Y., Kim, K.M., Waqas, M., et al. (2014) Silicon mitigates heavy metal stress by regulating P-type heavy metal ATPases., *Oryza sativa* low silicon genes, and endogenous phytohormones. BMC Plant Biol. 14: 13.

Koornneef, M., Reuling, G., Karssen, C.M. (1984) The isolation and characterization of abscisic acid-insensitive mutants of Arabidopsis thaliana. Physiol Plant 61: 377–383.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhard, N., Torres M.A., Dangl, J.L., et al. (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in arabidopsis. EMBO J. 22: 2623-2633.

Lau, O.S., Deng, X.W. (2010) Plant hormone signaling lightens up: integrators of light and hormones. Curr. Opin. Plant Biol. 13: 571-577.

Lei, G.J., Zhu, X.F., Wang, Z.W., Dong, F., Dong, N.Y. and Zheng, S.J. (2014) Abscisic acid alleviates iron deficiency by promoting root iron reutilization and transport from root to shoot in *Arabidopsis*. Plant Cell Environ. 37: 852-863.

Leon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., et al. (1996) Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. Plant J 10: 655–661.

Lequeux, H., Hermans, C., Lutts, S. and Verbruggen, N. (2010) Response to copper excess in *Arabidopsis thaliana*: Impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. Plant Physiol. Biochem. 48: 673-682.

Maksymiec, W, (2007) Signaling responses in plants to heavy metal stress. Acta Physiol. Plant. 29: 177-187.

Marschner, H. and Marschner, P. (2012) Marschner's mineral nutrition of higher plants. Academic press.

Nambara, E., Marion-Poll, A. (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56: 165–185.

Nakashima, K., Fujita, Y., Katsura, K., Maruyama, K., Narusaka, Y., Seki, M., et al. (2006) Transcriptional regulation of ABI3- and ABA-responsive genes including *RD29B* and *RD29A* in seeds, germinating embryos, and seedlings of *Arabidopsis*. Plant Mol. Biol. 60: 51-68.

Peñarrubia, L., Romero, P., Carrió-Seguí, A., Andrés-Bordería, A., Moreno, J. and Sanz, A (2015) Temporal aspects of copper homeostasis and its crosstalk with hormones. Front. Plant Sci. 6: 255.

Perales, L., Arbona, V., Gómez-Cadenas, A., Cornejo, M.J. and Sanz, A. (2005) A relationship between tolerance to dehydration of rice cell lines and ability for ABA synthesis under stress. Plant Physiol. Biochem. 43: 786-792.

Perea-García, A., Garcia-Molina, A., Andrés-Colás, N., Vera-Sirera, F., Pérez-Amador, M.A., Puig, S., et al. (2013) *Arabidopsis* copper transport protein COPT2 participates in the cross talk between iron deficiency responses and low-phosphate signaling. Plant Physiol. 162: 180-194.

Perea-García, A., Andrés-Bordería, A., Mayo de Andrés, S., Sanz, A., Davis, A.M., Davis, S.Jet al. (2016) Modulation of copper deficiency responses by diurnal and circadian rhythms in *Arabidopsis thaliana*. J. Exp. Bot. 67:391-403.

Peto, A., Lehotai, N., Lozano-Juste, J., León, J., Tari, I., Erdei, L., et al. (2011) Involvement of nitric oxide and auxin in signal transduction of copper-induced morphological responses in Arabidopsis seedlings. Ann. Bot. 108: 449-457.

Peuke, A.D., Jeschke, W.D. and Hartung, W. (2002) Flows of elements, ions and abscisic acid in *Ricinus communis* and site of nitrate reduction under potassium limitation. J. Exp. Bot. 53: 241-250.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucl. Acids Res. 29: e45.

Puig, S. (2014) Function and regulation of the plant COPT family of high-affinity copper transport proteins. Adv. Bot. 2014: 476917.

Romero, P., Rodrigo, M.J., Alférez, F., Ballester, A.R., González-Candelas, L., Zacarías, L., et al. (2012) Unravelling molecular responses to moderate dehydration in harvested fruit of sweet orange (*Citrus sinensis* L. Osbeck) using a fruit-specific ABA-deficient mutant. J. Exp. Bot. 63: 2753-2767.

Rubio, V., Bustos, R., Irigoyen, M.L., Cardona-López, X., Rojas-Triana, M. and Paz-Ares, J. (2009) Plant hormones and nutrient signaling. Plant Mol. Biol. 69: 361-373.

Saez, A., Robert, N., Maktabi, M.H., Schroeder, J.I., Serrano, R., Rodriguez, P.L. (2006) Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. Plant Physiol 141: 1389–1399.

Sancenón, V., Puig, S., Mateu-Andrés, I., Dorcey, E., Thiele, D.J. and Peñarrubia, L. (2004) The *Arabidopsis c*opper transporter COPT1 functions in root elongation and pollen development. J. Biol. Chem. 279: 15348-15355.

Sancenón, V., Puig, S., Mira, H., Thiele, D.J. and Peñarrubia, L. (2003) Identification of a copper transporter family in *Arabidopsis thaliana*. Plant Mol. Biol. 51: 577-587.

Sanchez, A., Shin, J. and Davis, S.J. (2011) Abiotic stress and the plant circadian clock. Plant Signal. Behav. 6: 223-231.

Schwarz, G. and Mendel, R.R. (2006) Molybdenum cofactor biosynthesis and molybdenum enzymes. Annu. Rev. Plant Biol. 57: 623-647.

Seki, M., Kamei, A., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr. Opin. Biotechnol. 14: 194-199.

Shi, W.G., Li, H., Liu, T.X., Polle, A., Peng, C.H. and Luo, Z.B. (2015) Exogenous abscisic acid alleviates zinc uptake and accumulation in Populus×canescens exposed to excess zinc. Plant Cell Environ. 38: 207-223.

Sommer, F., Kropat, J., Malasarn, D., Grossoehme, N.E., Chen, X., Giedroc D.P., et al. (2010) The CRR1 nutritional copper sensor in *Chlamydomonas* contains two distinct metal-responsive domains. Plant Cell 22: 4098-4113.

Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. Plant J. 11: 1187-1194.

Vysotskaya, L.B., Korobova, A.V. and Kudoyarova, G.R. (2008) Abscisic acid accumulation in the roots of nutrient-limited plants: Its impact on the differential growth of roots and shoots. J. Plant Physiol. 165: 1274-1279.

Waadt, R., Hitomi, K., Nishimura, N., Hitomi, C., Adams, S.R., Getzoff, E.D., et al. (2014) FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. eLife 2014;3:e01739.

Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I.D., et al. (2012) The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell Rep. 1: 639-647.

Xiong, L. and Zhu, J.K. (2002) Salt tolerance. The Arabidopsis Book 1: e0048.

Yamasaki, H., Hayashi, M., Fukazawa, M., Kobayashi, Y. and Shikanai, T. (2009) *SQUAMOSA Promoter Binding Protein–Like7* is a central regulator for copper homeostasis in *Arabidopsis*. Plant Cell 21: 347-361.

Yan, S. and Dong, X. (2014) Perception of the plant immune signal salicylic acid. Curr. Opin. Plant Biol. 20: 64-68.

Ye, N., Li, H., Zhu, G., Liu, Y., Liu, R., Xu, W., et al. (2014) Copper suppresses abscisic acid catabolism and catalase activity., and Inhibits seed germination of rice. Plant Cell Physiol. 55: 2008-2016.

Yruela, I. (2013) Transition metals in plant photosynthesis. Metallomics 5: 1090-1109.

Yuan, H.M., Xu, H.H., Liu, W.C. and Lu, Y.T. (2013) Copper regulates primary root elongation through PIN1-mediated auxin redistribution. Plant Cell Physiol. 54: 766-778.

Zhang, H., Zhao, X., Li, J., Cai, H., Deng, X.W. and Li, L. (2014) MicroRNA408 is critical for the HY5-SPL7 gene network that mediates coordinated response to light and copper. Plant Cell 26: 4933-4953.

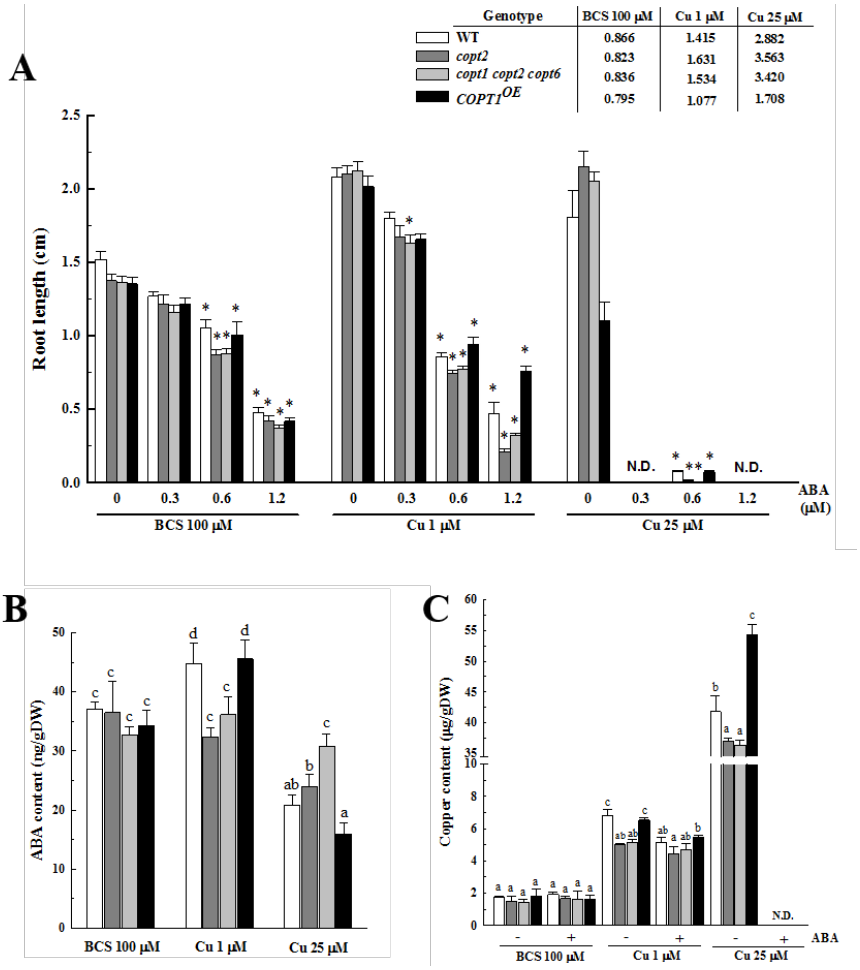


Figure 1. Sensitivity to ABA treatment in the *pmCOPT* mutants. In these experiments, the WT, *copt2*, *copt1 copt2 copt6* and *COPT1*^{OE} seedlings grown under Cu-deficiency (BCS 100 µM), Cu-sufficiency (Cu 1 µM) and Cu- excess (25 µM) conditions were used. (A) Root length of the 7-day-old plants grown in the presence of increasing ABA concentrations (0, 0.3, 0.6, 1.2 µM). Asterisks indicate statistical differences ($P<0.05$) according to Tukey's test with respect to the non ABA-treated (control) plants of each genotype under the same Cu condition. The slope values obtained by linear regression are shown in the legend. (B) ABA content in non ABA-treated WT, *copt2*, *copt1copt2copt6* and *COPT1*^{OE}. (C) Cu content in control and ABA-treated (0.6 µM) WT, *copt2*, *copt1 copt2 copt6* and *COPT1*^{OE}. Bars are means ± SD of 3 replicates of at least 10 plants (A) or of 200 mg FW (B and C) each. In C, each Cu condition was analyzed separately. In A and C, N.D. indicates no data.

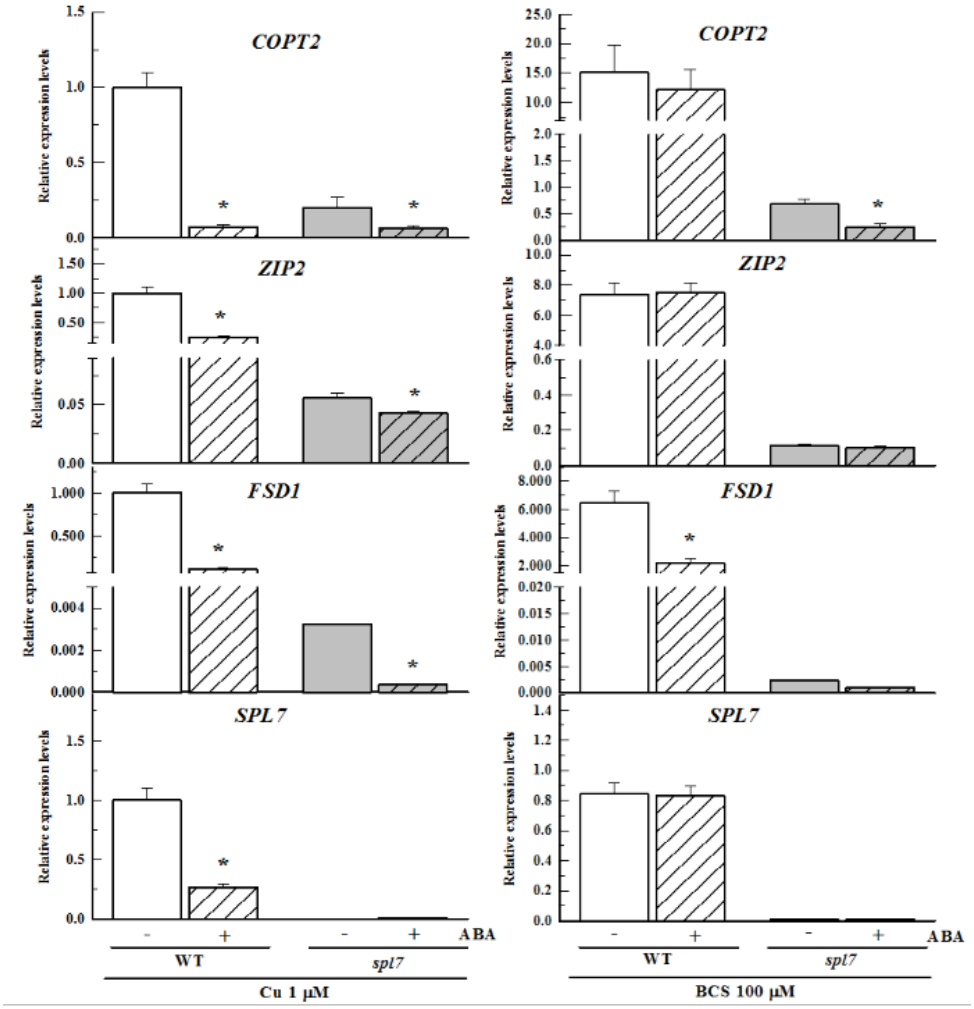


Figure 2. Effect of ABA treatment on the transcriptional regulation of Cu homeostasis-related genes. Transcript levels of genes *COPT2*, *ZIP2*, *FSD1* and *SPL7* in the WT and *spl7* seedlings, treated or not with ABA (0.6 µM), and grown under Cu-sufficiency (A) and Cu-deficiency (B) conditions. Expression values are relative to those reported in the non ABA-treated (control) WT seedlings grown under the Cu-sufficiency conditions per gene. Bars correspond to arithmetic means ($2^{-\Delta\Delta Ct}$) ± standard deviation (SD) (*n*=3). For each particular gene, asterisks indicate statistical differences ($P<0.05$) with values of the control plants of each genotype.

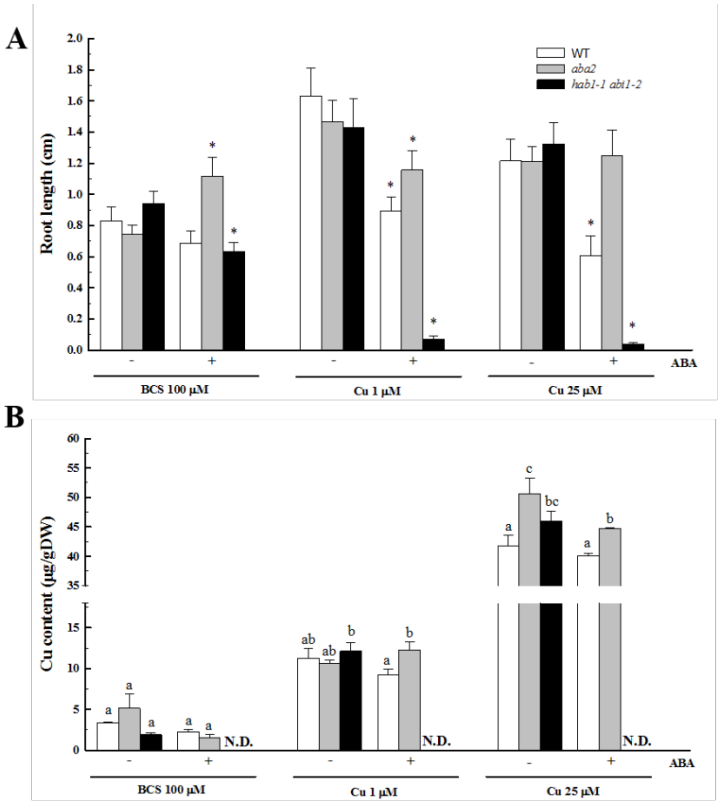


Figure 3. Sensitivity to Cu treatment in the *ABA* mutants. In these experiments, the WT, *aba2* and *hab1-1 abi1-2* seedlings grown under Cu-deficiency (BCS 100 µM), Cu-sufficiency (Cu 1 µM) and Cu- excess (25 µM) conditions were used. (A) Root length of the 7-day-old plants grown in the presence of 0.6 µM ABA. Asterisks indicate statistical differences ($P<0.05$) according to Tukey's test with respect to the non ABA-treated (control) plants of each genotype under the same Cu condition. (B) Cu content in both ABA-treated (0.6 µM) and non ABA-treated WT, *aba2* and *hab1abi1*. N.D. indicates no data. Bars are means ± SD of 3 replicates of at least 15 plants (A) or of 200 mg FW (B) each. In B, each Cu condition was analyzed separately.

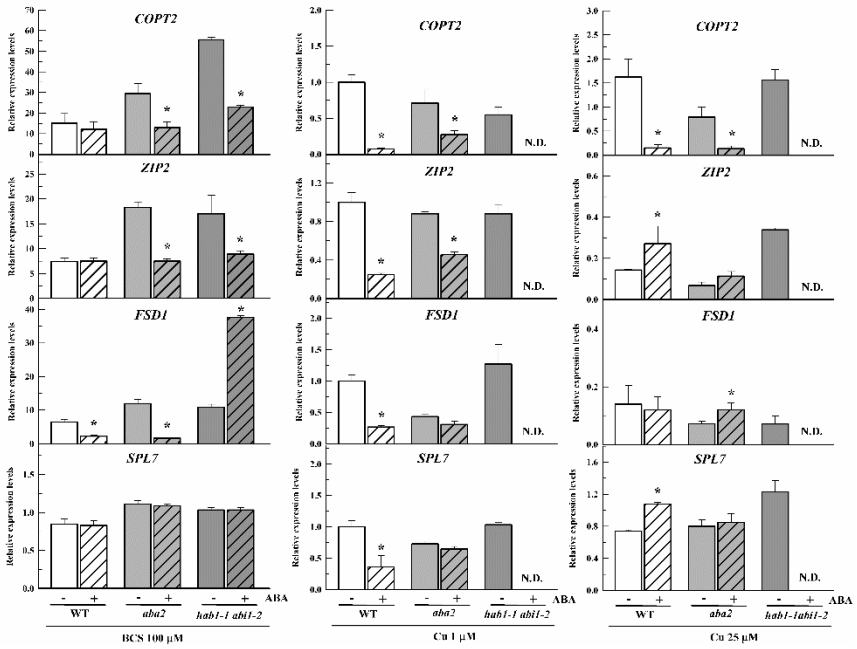


Figure 4. Effect of Cu treatment in ABA mutants on the transcriptional regulation of Cu homeostasis-related genes. Transcript levels of genes *COPT2*, *ZIP2*, *FSD1* and *SPL7* in the WT, *aba2* and *hab1-1 abi1-2* seedlings, treated or not with ABA (0.6 µM) and grown under Cu-deficiency (A), Cu-sufficiency (B) and (C) Cu-excess conditions. Expression values are relative to those reported in the non ABA-treated (control) WT seedlings grown under the Cu-sufficiency conditions per gene. N.D. indicates no data. Bars correspond to arithmetic means ($2^{-\Delta\Delta Ct}$) ± standard deviation (SD) (n=3). For each particular gene, asterisks indicate statistical differences ($P<0.05$) with values of the control plants of each genotype.

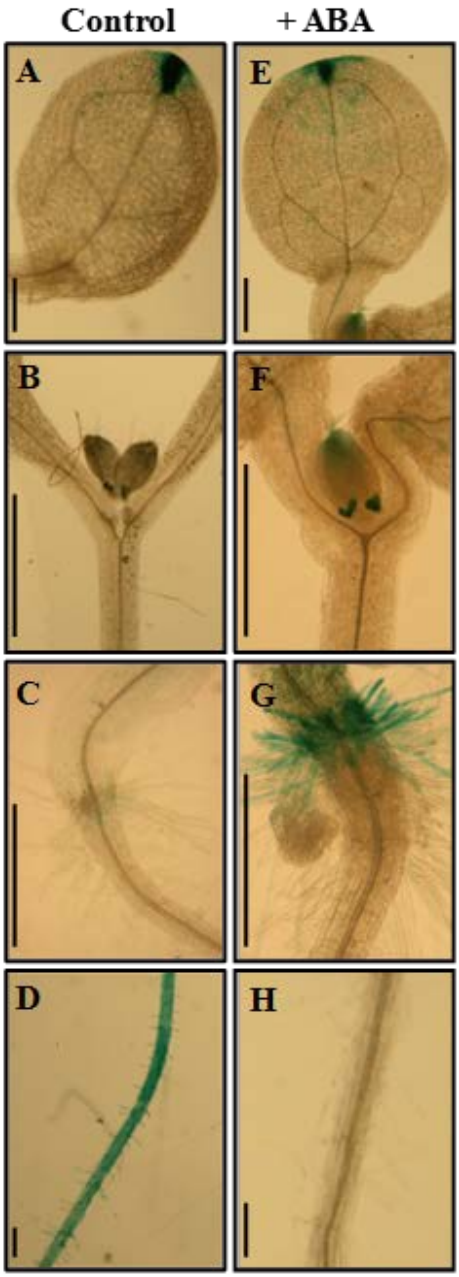


Figure 5. Effect of ABA treatment on the spatial *COPT2* gene expression pattern. *COPT2* expression pattern in the *Arabidopsis pCOPT2::GUS* transgenic seedlings treated (+ABA) or not (control) with ABA (0.6 µM), and grown under mild Cu deficiency (½ MS). GUS staining is shown in a representative 7-day-old seedling cotyledon (A, E), detail of shoot meristem and trichomes (B, F), root crown (C, G) and a portion of root tissues (D, H). GUS reaction was performed at 6 h from the onset of the light period. Bars indicate 1 cm in each particular photograph.

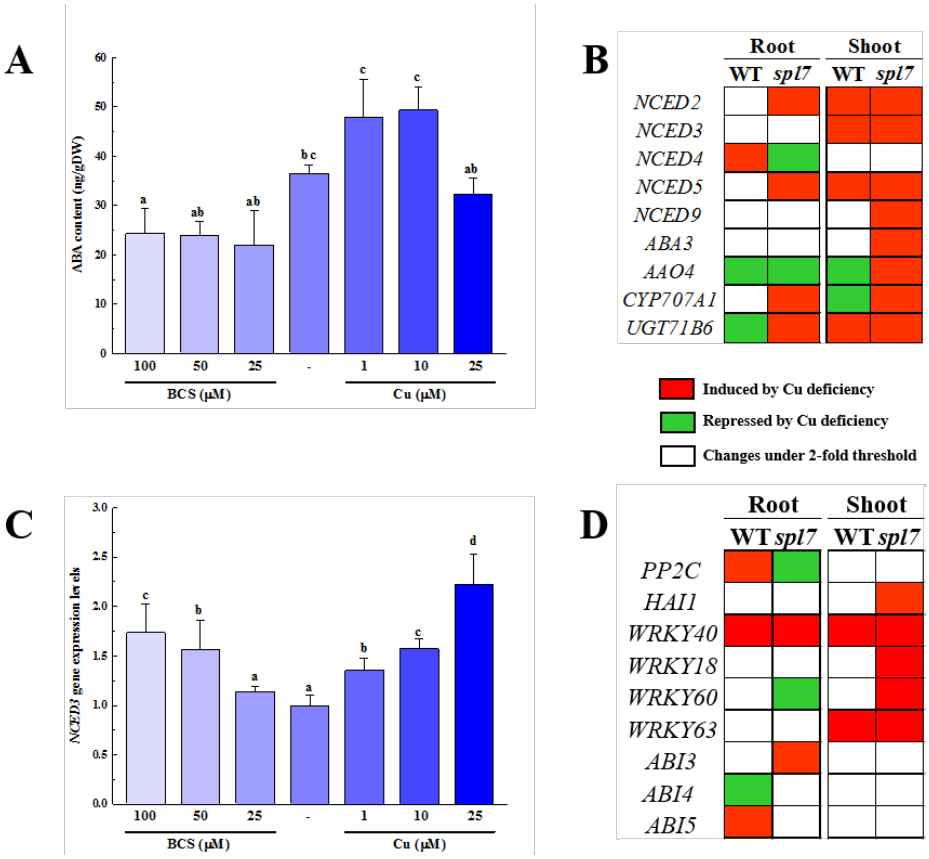


Figure 6. Effect of Cu availability on ABA accumulation. (A) ABA content in the 7-day-old WT seedlings grown on a Cu gradient scale, which ranged from severe deficiency (100 µM BCS) to moderate excess (25 µM $CuSO_4$). Bars are means ± SD of 3 independent replicates of 200 mg FW each. Different letters indicate statistical differences ($P<0.05$) according to Tukey's test. (B) ABA biosynthetic genes differentially expressed under Cu deficiency, as reported by Bernal et al. 2012. Colors indicate at least a 2-fold induction (red), repression (green) or less than 2-fold change (white) in the plants grown under the Cu deficiency *vs.* optimal Cu conditions. Analyses were performed on root and shoot tissues from the WT and *spl7* mutant plants. NCED: nine-*cis*-epoxycarotenoid dioxygenase; ABA3: ABA deficient 3; AAO: ABA aldehyde oxidase; CYP707A: ABA 8'-hydroxylases; UGT71B6: UDP-glucosyltransferase. (C) *NCED3* gene expression levels in the WT seedlings grown as indicated in panel A. Transcript levels are relative to those reported in the ½ MS samples. Bars are means±SD of 3 replicates of 200 mg FW each. Different letters indicate statistical differences ($P<0.05$) according to Tukey's test. (D) ABA signaling genes

differentially expressed under Cu deficiency, as reported by Bernal et al. 2012. Key of colors as described in (B). PP2CAs: clade A protein phosphatases 2C; WRKY: transcription factors that contain the WRKY DNA-binding domain; ABI: Abscisic acid insensitive.

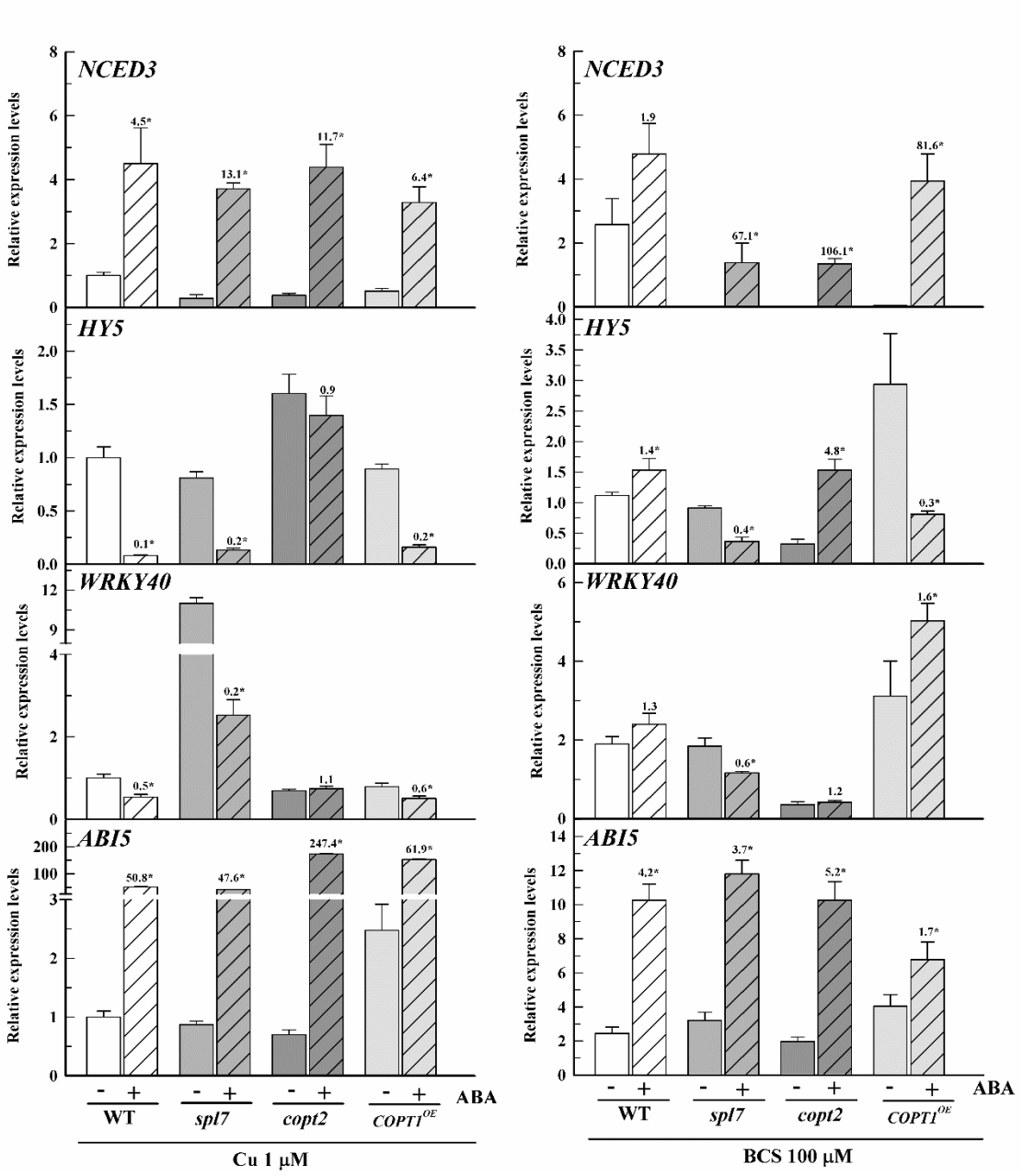


Figure 7. Effect of Cu availability on the transcriptional regulation of the ABA-related genes. Transcript levels of genes *NCED3*, *HY5*, *WRKY40* and *ABI5* in WT, *spl7*, *copt2* and *COPT1*^{OE} in the 7-day-old seedlings treated or not with ABA (0.6 µM), grown under Cu-sufficiency (A) and Cu-deficiency (B) conditions. Expression values ($2^{-\Delta\Delta Ct}$) are relative to those reported in the control WT seedlings. Bars are means ± SD of 3 replicates of at least 10 plants each. Numbers indicate the fold

changes in the transcript levels of the ABA-treated with respect to control samples per genotype. Asterisks indicate statistical differences (P<0.05).

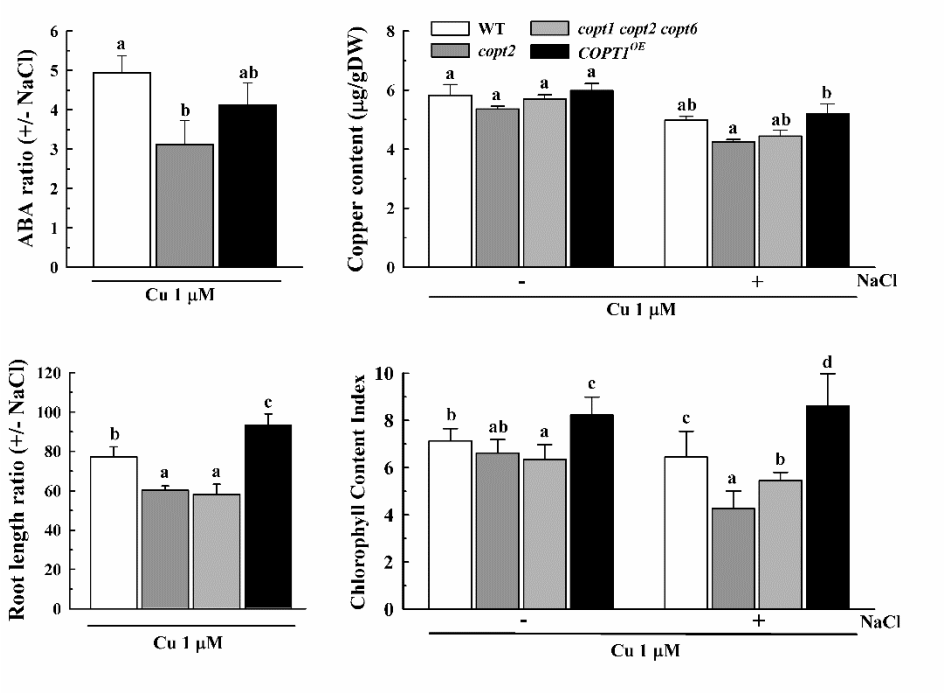
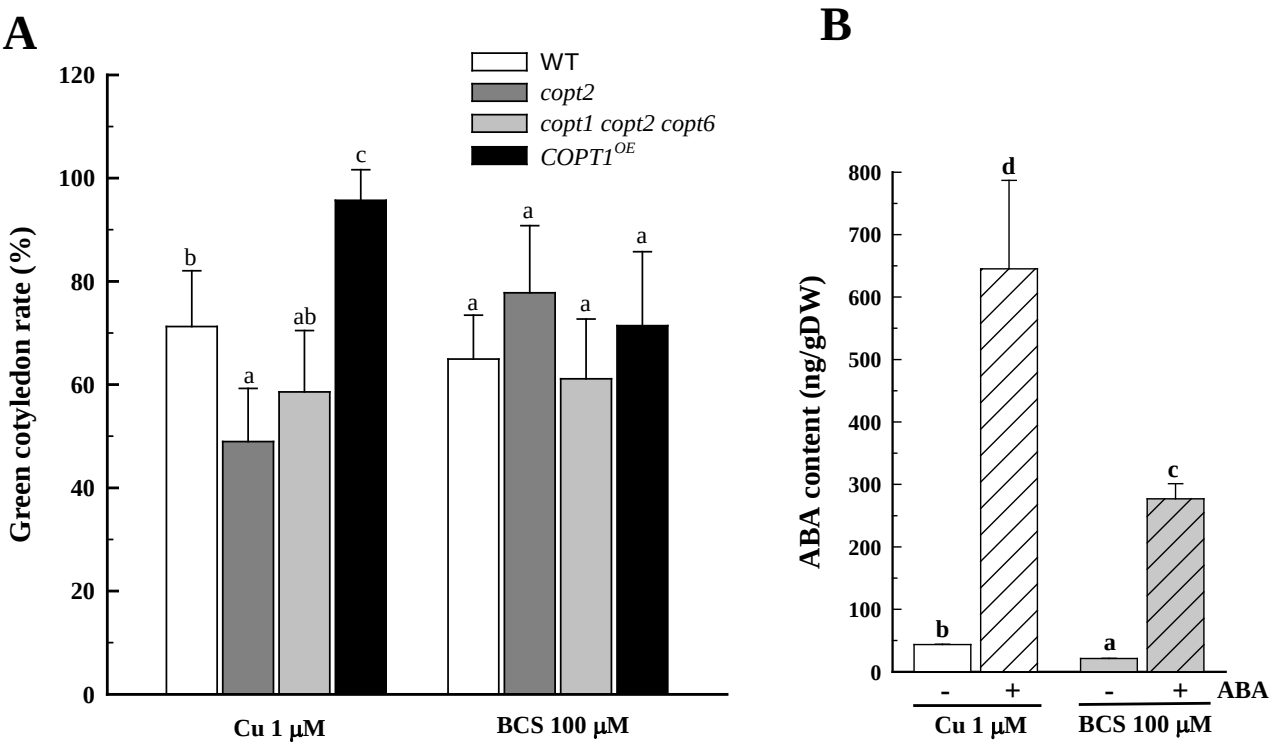
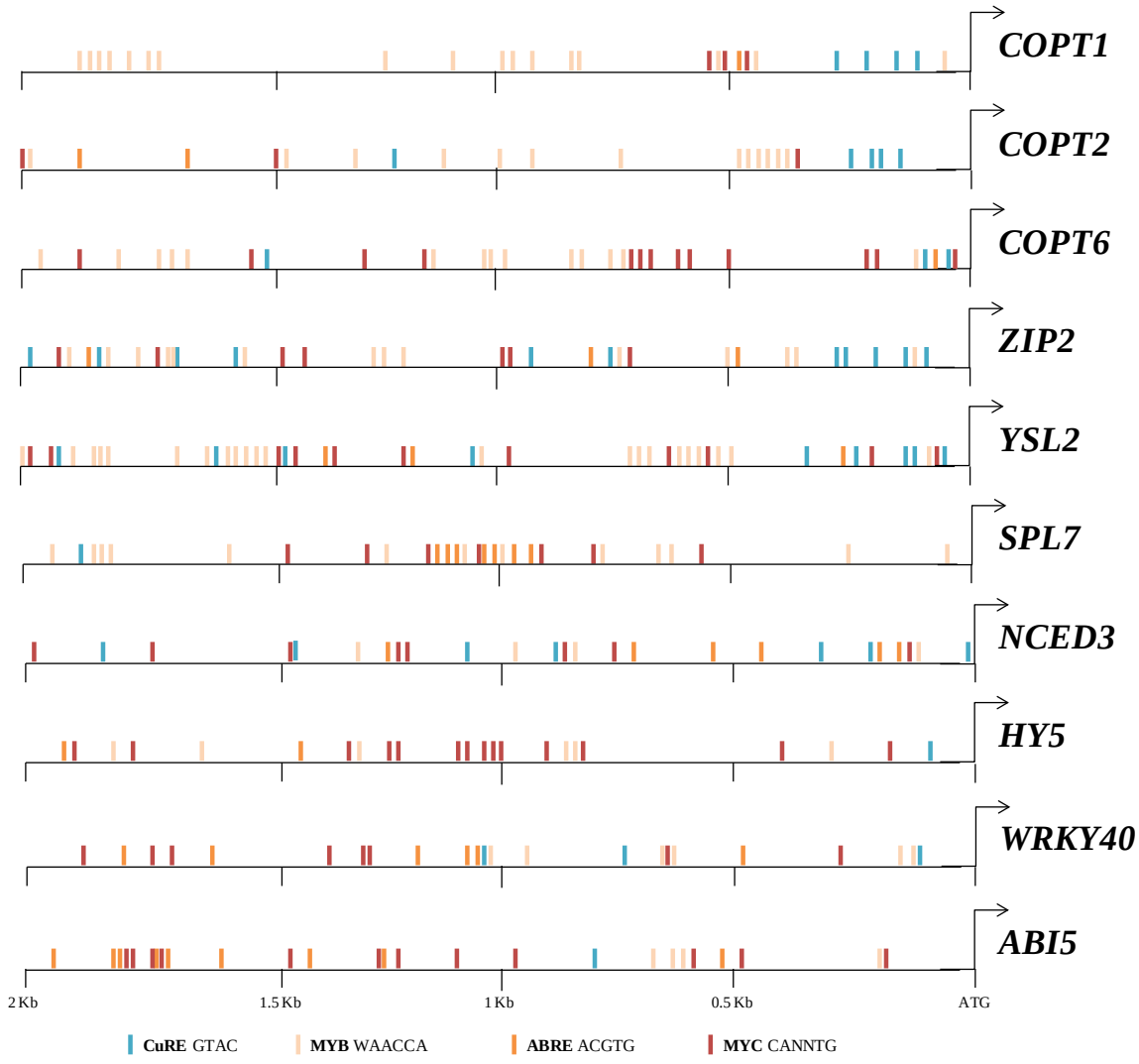


Figure 8. Sensitivity to salt stress in the knockout and overexpressing *pmCOPT*s mutants under Cu-sufficiency conditions. The control and salt-stressed (200 mM NaCl) WT, *copt2*, *copt1 copt2 copt6* and *COPT1*^{OE} 14-day-old seedlings, grown under Cu-sufficiency conditions, were used to determine the ABA content ratio (A), Cu content (B), root length ratio (C) and chlorophyll content index (D). Bars are means ± SD of 3 replicates of at least 200 mg FW. Different letters indicate statistical differences (P<0.05) according to Tukey's test for each particular condition.

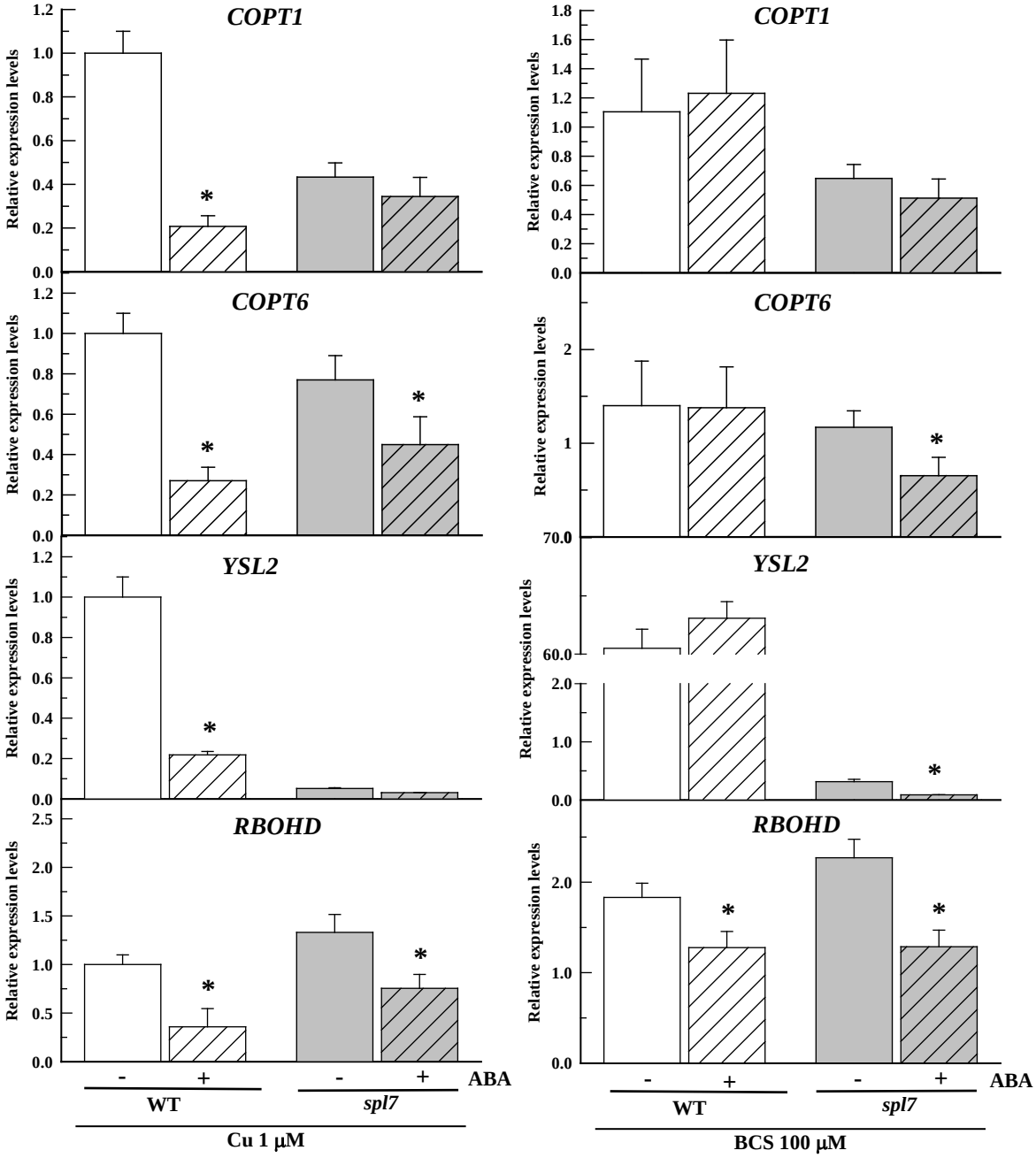
SUPPLEMENTARY MATERIAL



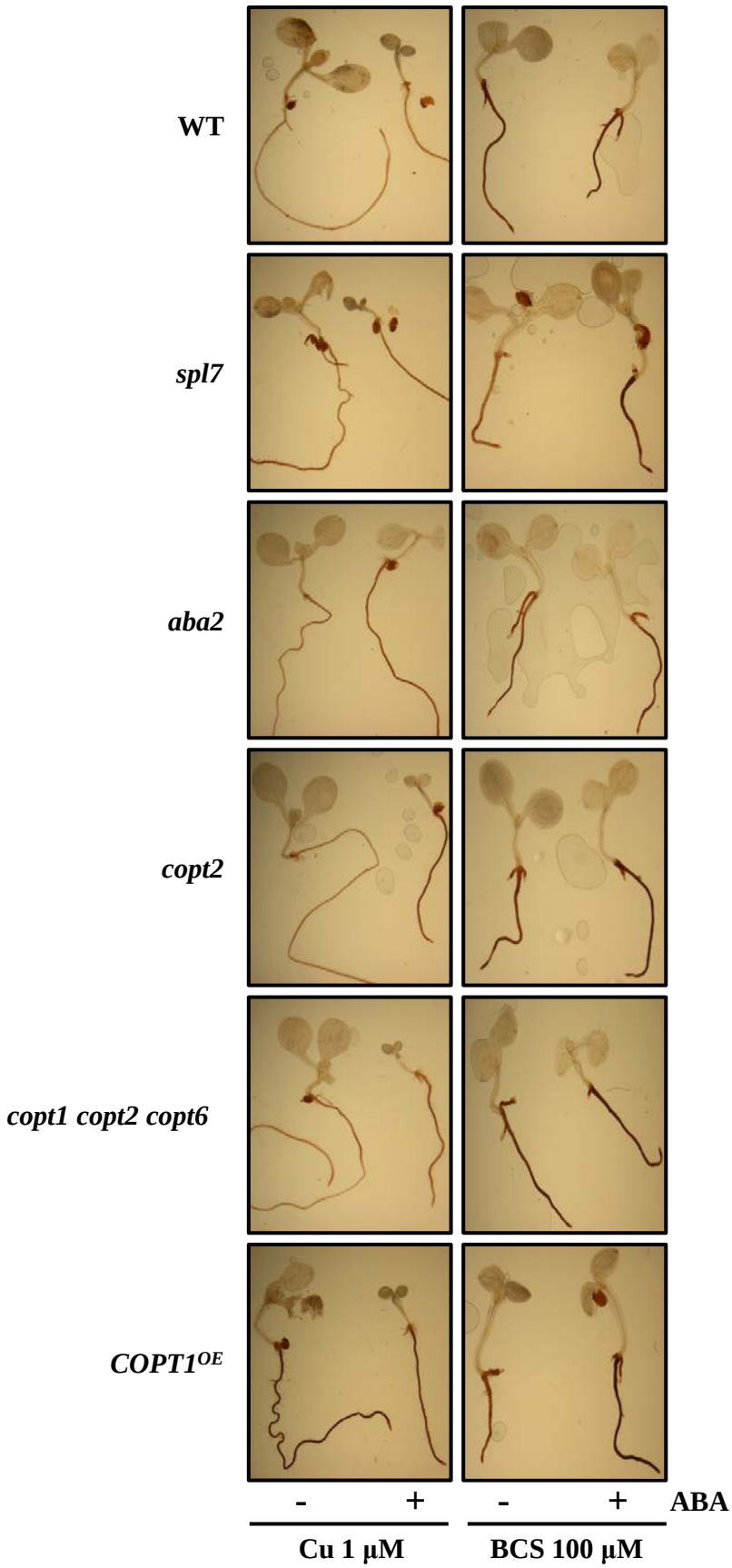
Supplementary Figure S1.- Sensitivity to exogenous ABA. (A) Green cotyledon rate (GCR) measured in the presence of 0.6 µM ABA. Values for the control plants were 100% in all genotypes. Different letters indicate statistical differences (P<0.05) according to two-tailed z-tests for each Cu condition. (B) ABA content after 7 days of 0.6 µM ABA treatment applied to WT seedlings. Different letters indicate statistical differences (P<0.05) according to Tukey's test.



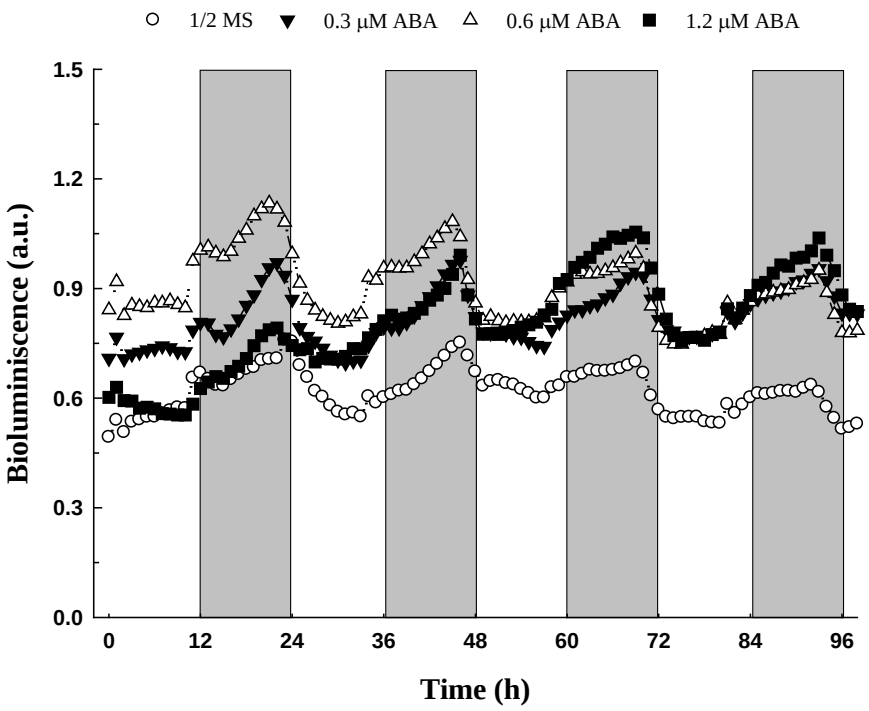
Supplementary Figure S2. Analysis of putative ABF and SPL7 recognized sites in the promoter sequences of ABA- and Cu deficiency-related genes. Positions of the *cis*-regulatory elements are marked in different colors as indicated in the figure legend. Promoter regions were defined as the 2000 bp upstream of the five prime untranslated region (5′-UTR) from the corresponding genes. The *in silico* analysis of the *cis*-elements present in the promoter regions was performed in the PLACE database.



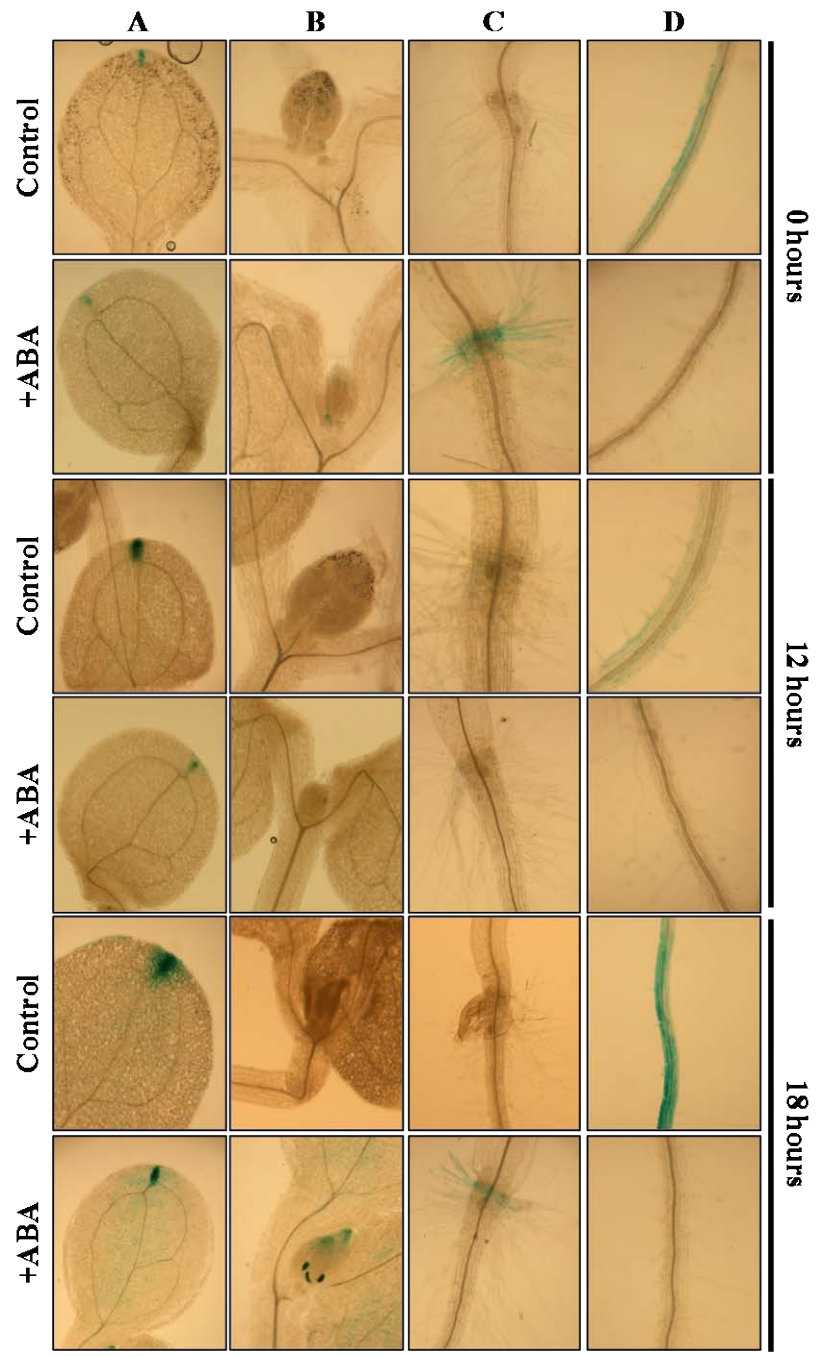
Supplementary Figure S3. Effect of ABA treatment on the transcriptional regulation of Cu homeostasis-related genes. Transcript levels of genes *COPT1*, *COPT6*, *YSL2* and *RBOHD* in the WT and *spl7* seedlings, treated or not with ABA (0.6 μM), and grown under Cu-sufficiency **(A)** and Cu-deficiency **(B)** conditions. Expression values are relative to those reported in the non ABA-treated (control) WT seedlings grown under the Cu-sufficiency conditions per gene. Bars are the means ± SD of 3 replicates of at least 10 plants each. For each particular gene, asterisks indicate statistical differences ($P<0.05$) with the control plants of each genotype.



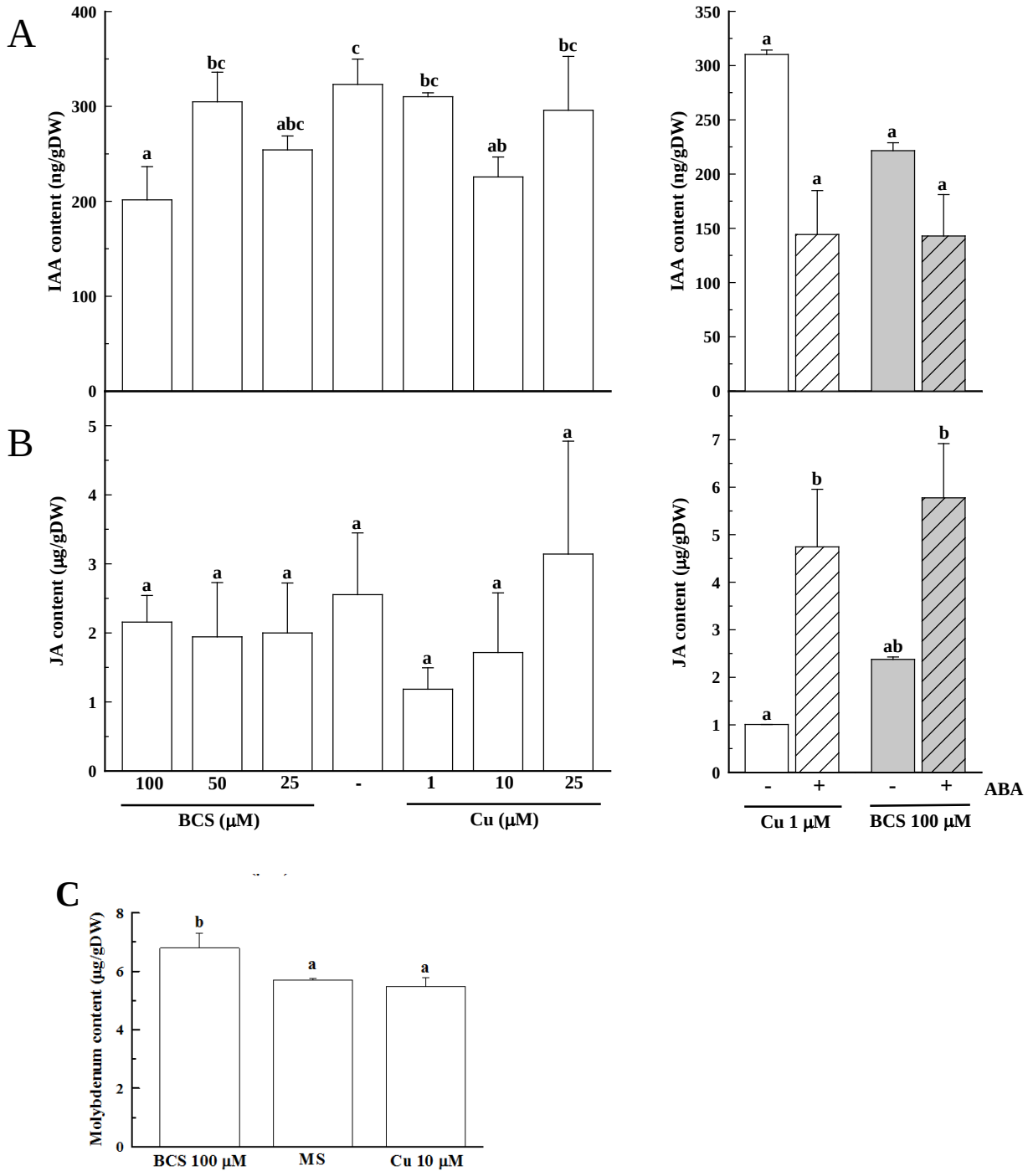
Supplementary Figure S4. Effect of ABA and Cu availability on oxidative stress status. Hydrogen peroxide detection in WT, *spl7*, *aba2*, *copt2*, *copt1 copt2 copt6* and *C1*^{OE} 7 day-old *Arabidopsis* seedlings grown under Cu sufficiency and deficiency conditions treated (+) or not (-) with ABA (0.6 µM, 7 days). Shown is DAB staining in a representative 7 day-old seedling for each genotype and condition.



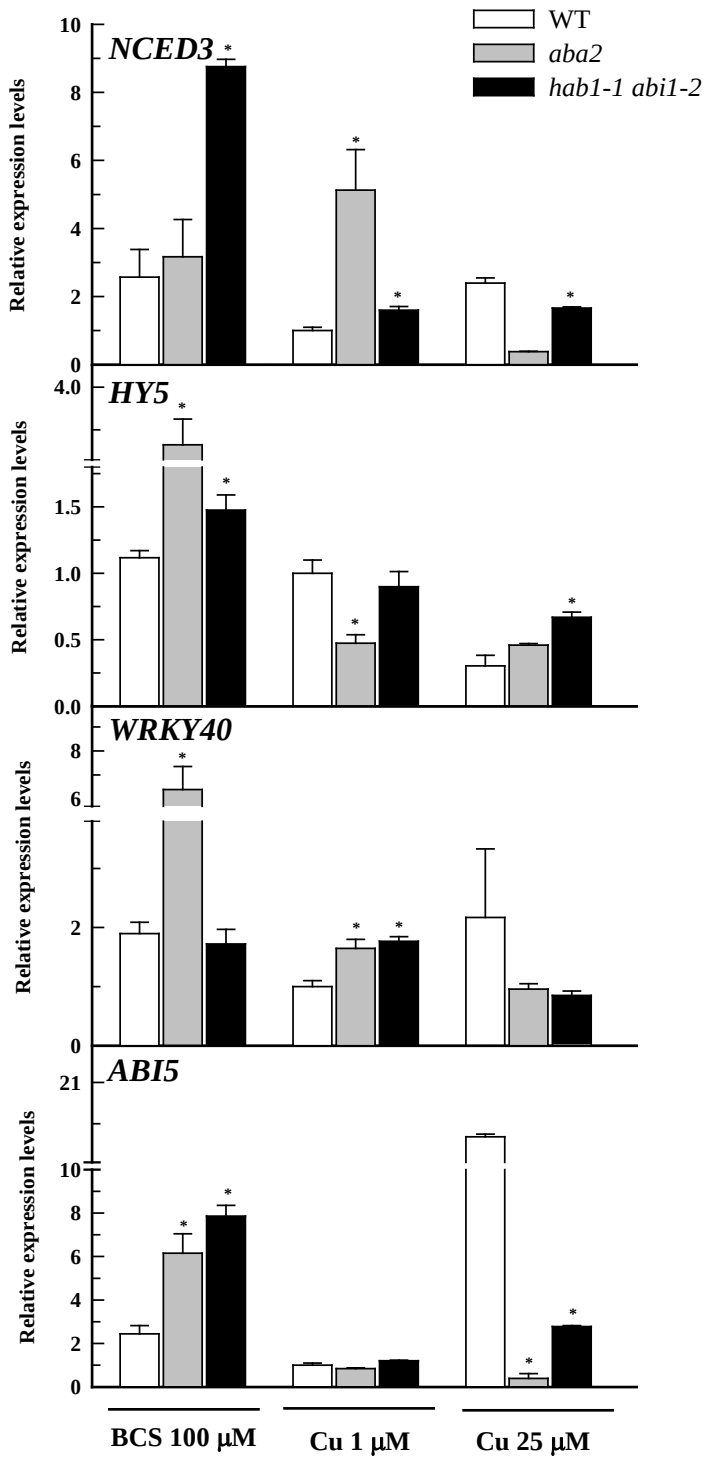
Supplementary Figure S5. Effect of ABA on the oscillatory *COPT2* gene expression pattern. *COPT2* expression pattern in *Arabidopsis pCOPT2::LUC* transgenic seedlings treated with different ABA concentrations and grown under mild Cu deficiency (½ MS). Shown is luciferase activity measured as bioluminescence emission.



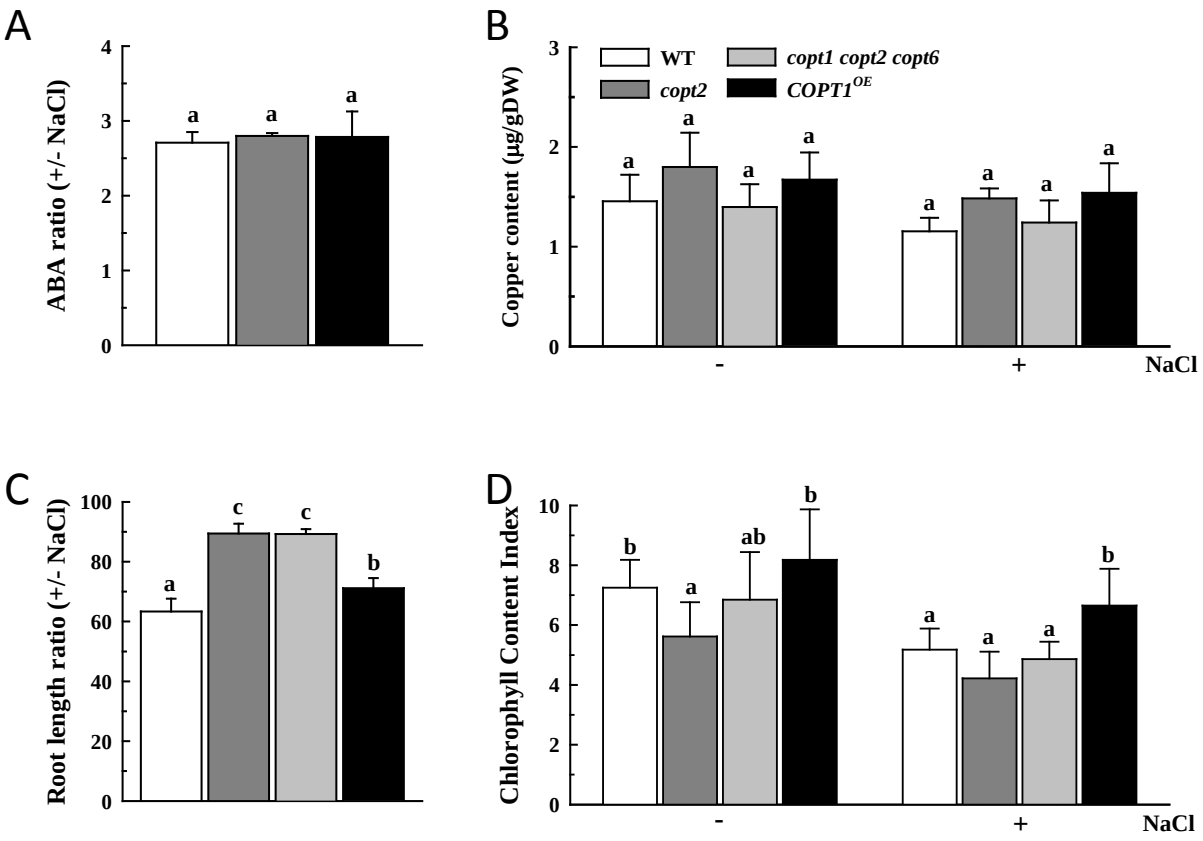
Supplementary Figure S6. Effect of ABA on the spatial *COPT2* gene expression pattern. *COPT2* promoter-driven expression pattern in *Arabidopsis pCOPT2::GUS* transgenic seedlings treated (+ABA) or not (Control) with ABA (0.6 μM) and grown under mild Cu deficiency (½ MS) conditions. A representative 7 day-old seedling cotyledon (A), detail of shoot meristem and trichomes (B), root crown (C) and a portion of root tissue (D) is shown for GUS staining. GUS reaction was performed at 0, 12 and 18 h from the onset of the light period.



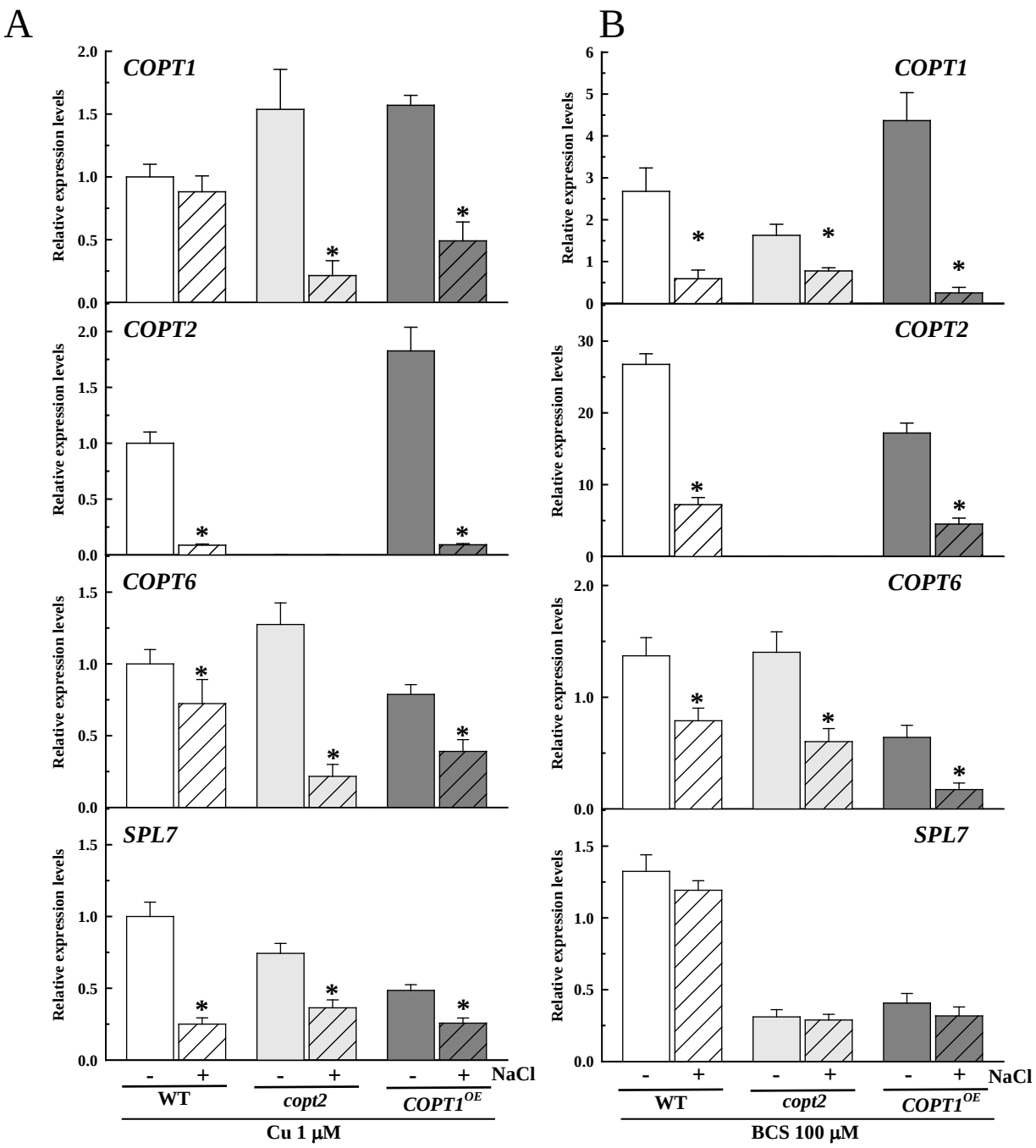
Supplementary Figure S7. Effect of Cu availability and ABA on hormone accumulation. (**A**) IAA and JA content were measured in 7 day-old WT seedlings grown under a Cu range from severe deficiency (BCS 100 µM) to Cu excess (25 µM). (**B**) IAA and JA levels measured in control and ABA-treated (0.6 µM) 7 day-old WT seedlings grown under Cu sufficiency (Cu 1 µM) and severe Cu deficiency (BCS 100 µM). (**C**) Molybdenum content in WT seedlings grown under different Cu availability conditions. Bars are means ± SD of 3 biological replicates of 200 mg FW. Different letters indicate statistical differences ($P<0.05$) according to a Tukey's test.



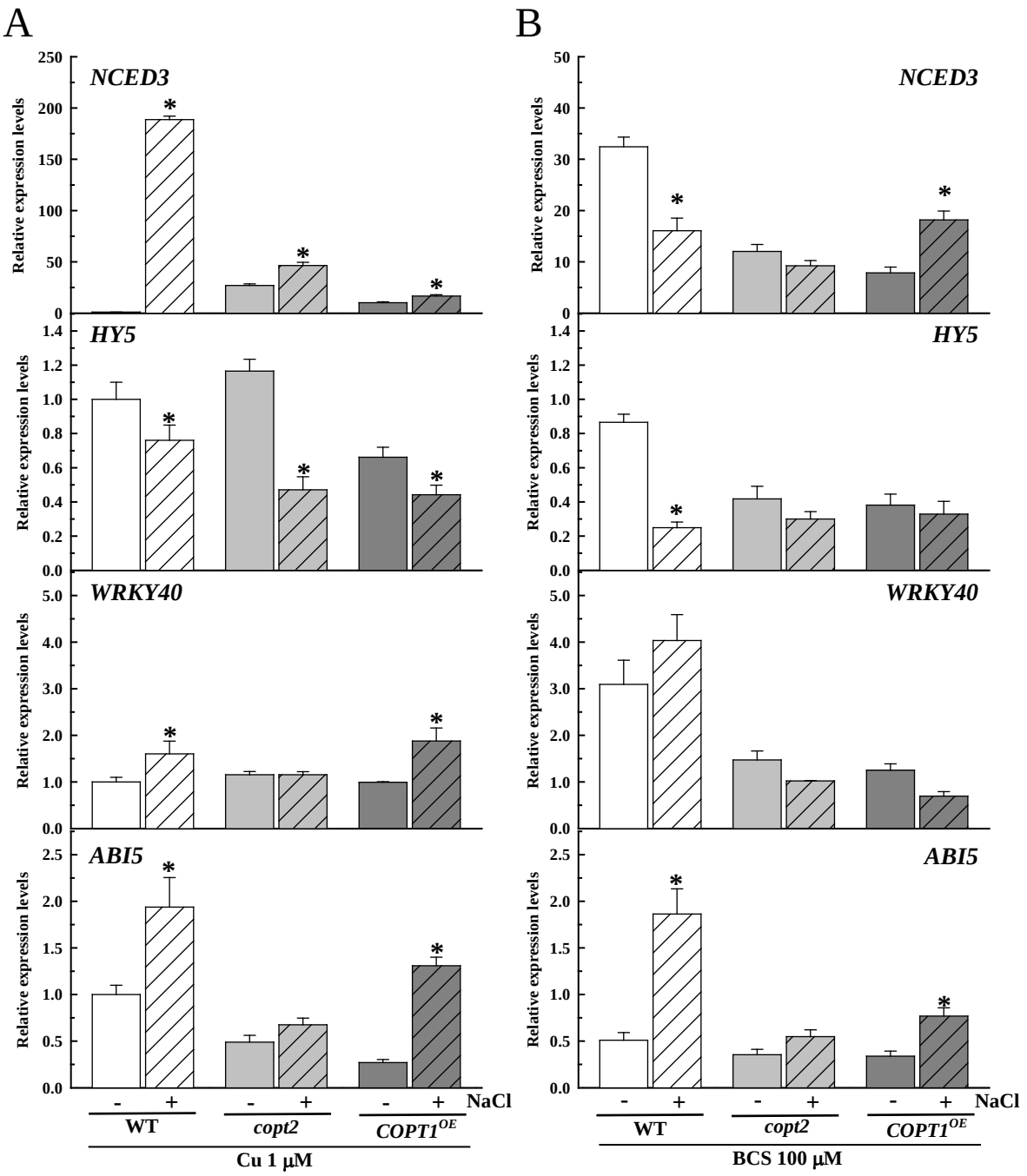
Supplementary Figure S8. Effect of Cu availability on the transcriptional regulation of ABA-related genes in ABA mutants. Transcript levels of genes *NCED3*, *HY5*, *WRKY40* and *ABI5* in WT, aba*2* and *hab1-1 abi1-2* in the 7-day-old seedlings grown under Cu-deficiency (BCS 100 µM), Cu-sufficiency (Cu 1 µM) and Cu-excess (Cu 25 µM) conditions. Expression values arerelative to those reported in the control WT seedlings. Values correspond to arithmetic means ($2^{-\Delta\Delta Ct}$) ± standard deviation (SD) (*n*=3). Asterisks indicate statistical differences ($P<0.05$).



Supplementary Figure S9. Sensitivity to salt stress in knockout and overexpressing *pmCOPT*s mutants under Cu deficiency conditions. Measurements were performed in 14 day-old WT, *copt2* and *COPT1*^{OE} seedlings grown under Cu deficiency which were salt stressed (200 mM NaCl) during the last 7 days. In (B) and (D) the triple mutant *copt1 copt2 copt6* was also studied. **(A)** ABA content ratio between salt-stressed and control seedlings. **(B)** Cu content in control and salt-stressed seedlings. **(C)** Root length ratio between salt-stressed and control seedlings. **(D)** Chlorophyll content index in control and salt-stressed seedlings. Bars are means of 3 replicates of at least 200 mg FW. Different letters indicate statistical differences ($P<0.05$) according to Tukey's tests for each particular condition.



Supplementary Figure S10. Effect of salt stress on *COPT*s and *SPL7* transcriptional regulation. Transcript levels of *COPT1*, *COPT2*, *COPT6* and *SPL7* genes in WT, *copt2*, and *COPT1*^{OE} 14 day-old seedlings treated or not with NaCl (200 mM, 7 days) and grown under Cu sufficiency **(A)** and deficiency **(B)** conditions. Expression values are relative to those reported in non NaCl-treated (control) WT seedlings grown under Cu sufficiency conditions for each gene. Bars are means ± SD of 3 replicates of at least 10 plants each. Asterisks indicate statistical differences ($P<0.05$) according to Tukey's test respect to values of control plants of each genotype.



Supplementary Figure S11. Effect of salt stress on the transcriptional regulation of ABA-related genes. Transcript levels of *NCED3*, *HY5*, *WRKY40* and *ABI5* genes in WT, *copt2*, and *COPT1*^{OE} 14 day-old seedlings treated or not with NaCl (200 mM, 7 days) and grown under Cu sufficiency **(A)** and deficiency **(B)** conditions. Expression values are relative to those reported in non NaCl-treated (control) WT seedlings grown under Cu sufficiency conditions for each gene. Bars are means ± SD of 3 replicates of at least 10 plants each. Asterisks indicate statistical differences ($P<0.05$) according to Tukey's test respect to values of control plants of each genotype.

Supplementary Table S1. Selected genes and primers used for qRT-PCR analyses.

GENE	FORWARD (5'→3')	REVERSE (5'→3')
ABI5	ACTTCCAGCTCCGCTTTGTA	GGTTGTCTAGCCGCAGTCTC
COPT1	TTGCAATTTTCCTCTCCTCCCAA	ATGATGGTCGAGGCATT
COPT2	CCTTTCGTATTTGGTGATGCT	AAACACCTGCGTTAAAGGAC
COPT6	GCAGTCTACACACTCAAGACA	AAAGGACATAACGGCGAGCA
FSD1	ACCGAAGACCAGATTACATA	TGGCACTTACAGCTTCCCAA
HY5	GTTTGGAGGAGAAGCTGTCG	TCTTGCTTGCTGAGCTGAAA
NCED3	ATTGGCTATGTCGGAGGATG	CGACGTCCGGTGATTTAGTT
RBOHD	CGAGGAGCTAACTCGGACAC	GCGTGATCTCCACGTACTCA
SPL7	AGTTTGACGGGACCTGAATG	AGTTTGACGGGACCTGAATG
UBQ10	TAATCCCTGATGAATAAGTGTTCTAC	AAAACGAAGCGATGATAAAGAAG
WRKY40	GTGGAGGATCAGTCCGTGTT	TCTGAACTTGGGGAAAATCG
YSL2	TCTTATAAATGGATTTCATACTA	AATGCCCAAAAGAAACTCAAA
ZIP2	CGCTTGGAGAAACCTATGGA	CGACACCTATGGGACTCGAT