Gene expression profiling analysis of copper homeostasis in *Arabidopsis thaliana*

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ABSTRACT

As a result of copper essentiality for life, plants and most other organisms have developed a conserved and complex network of proteins to handling Cu in order to prevent its deficit and to avoid its potentially toxic effects. To better understand regulation of Cu homeostasis in plants, we use adult plant of *Arabidopsis thaliana* to provide an integrated view of how Cu status affects the expression of genes involved in cellular Cu homoeostasis. In doing so, we use real-time RT-PCR to compare shoot and roots transcriptional responses to Cu. We measure changes in the abundance of transcripts encoding transporters, chaperones and P-type ATPases and correlated those changes with variation of Cu content in both tissues. Our results indicated that in both tissues transcript levels of *COPT2*, *4*, and *ZIP2* transporters and *CCH* chaperone were significantly down-regulated comparing to controls plants in response to Cu excess. In contrast, Cu chaperones *ATX1*, *CCS*, *COX17-1* including two putative mitochondrial chaperones (At3g08950; At1g02410) were up-regulated under similar conditions. Regarding P-type ATPases, a reduction of *HMA1*, *PAA1*, *PAA2*, and *RAN1* transcript levels in shoot after Cu exposure was observed, while *HMA5* transcripts increased exclusively in roots. In plants growing under Cu-deficient conditions, *COPT2*, *ZIP2*, *HMA1, and PAA2*, were significantly up-regulated in shoots. Thus, our results indicated a common transcriptional regulation pattern of transporters and chaperone components, in particular transcriptional changes of *COPT2*, *ZIP2*, and *CCH* showed an inverse relation with Cu content suggesting that these proteins are required to avoid excess and deficit of Cu.

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Introduction

Copper (Cu) is an essential micronutrient for life. It is required by a wide range of species, from bacteria to yeast, plants and mammals including human. In plants Cu plays an important role as a cofactor in several metabolic processes, including photosynthetic and mitochondrial electron transport, oxidative stress responses, and hormone perception [1]. Thus, Cu is an essential micronutrient for normal growth and development and deficiency of this metal has a severe effect on pollen development and viability, fruit and seed production [2]. Nevertheless, the intracellular Cu level must be tightly regulated, since it is toxic for most plants when is present in excess. The reactivity of Cu ions can lead to the generation of harmful reactive oxygen species via Fenton and Haber–Weiss reactions that cause severe oxidative damage to cells [3]. Cu excess causes phytotoxicity by inhibiting important cellular processes, including photosynthesis and electron transport, lipid peroxidation, and disruption of protein functions due to Cu-binding to sulphydryl groups [4,5].

Thus to prevent the damage of Cu excess and to respond to Cu deficiency, plants have evolved cellular and molecular mechanisms to maintain Cu homeostasis. The strategies that plants use to maintain Cu homeostasis include the regulation of Cu uptake in root cells [6], Cu trafficking via P-type ATPases and Cu chaperones [7–9] and regulation of the levels of Cu proteins in response to a change in metal availability [10]. Several proteins or families of proteins involved in plant homeostasis are largely conserved trough the diversity of living organisms [11]. Thus, studies in yeast have revealed that free Cu concentrations in the cytosol are strictly regulated by a complex Cu homeostasis system, consisting of Cu transporters (Ccc2p), Cu-binding proteins (Cox17p) and Cu chaperone (Ccsp) which display amino acid sequence homology and functional properties analogous to their plant counterparts [11]. Despite the importance of preventing both Cu deficiency and toxicity in plants still much remains to be elucidated about the mechanisms controlling Cu homeostasis and trafficking inside the cell. In this work we aim to understand how plants adapt to changes in copper availability by the simultaneous examination of transcript levels of genes encoding key components of Cu homeostasis in *Arabidopsis thaliana*. In doing so, we used real-time RT-PCR as a sensitive method to measure changes in the relative abundance of transcripts. Our results indicated that genes belonging

to the three functional categories analyzed (transporters, chaperones and P-type ATPases) changed their expression levels in response to copper availability. In particular, genes encoding *COPT2*, *ZIP2* uptake transporters and *CCH* chaperone displayed a common transcriptional regulation pattern in both shoot and root. They showed an inverse relation with Cu content suggesting that their encoded proteins are required to avoid excess and deficit of Cu.

Materials and methods

Plant material and Cu treatments. Arabidopsis thaliana (L) ecotype Columbia (0) seeds were sterilized for 8 min with 15% (v/v) household bleach containing a few drops of Tween 20, followed by three washes with sterile water and then placed on water/agar (0.8% w/v) containing yellow tip as described by Noren et al. [12]. After vernalisation at $4\,°C$ for 2 days, seeds were transferred to a growth cabinet and then grown hydroponically in a standard medium (containing 1.25 mM KNO_3, 0.62 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.5 mM, $Ca(NO_3)$, 42.5 μM Sequestrene and micronutrients excluding Cu; adapted from Arteca and Arteca [13], with weekly replacement of the hydroponic medium. We used adult plants when they had developed a full rosette but not yet started flowering. For Cu-deficient condition, standard medium was replaced with medium containing 50 μM of bathocuprein (BCS) a Cu-specific chelator [14]. The plants were grown in this medium for five days and then harvested. For Cu treatments, standard medium was supplemented with 50 μm $CuSO_4$ and plants were harvested at several time points after treatment. Control plants (no Cu or BCS supplemental) were transferred to fresh standard medium at the onset of the respective treatments and they were harvested at the same time as treated plants. In all experiments, 50–70 individuals were pooled from each condition. Plants were grown at $22\,°C$ with a 16-h/8-h light/ dark photoperiod (140 μmol/m^2s^1).

Quantification of Cu. After Cu treatments, tissues were collected and roots were washed once with buffer MES-KOH pH 5.5 containing EDTA 5 mM and $CaCl_2$ 0.5 mM and then three times in deionized water. Metal content analysis was carried out on dried root and shoot (seven pooled plants by treatment). To obtain dry weight (DW), tissues were dried at $65\,°C$ for 72 h. After acid extraction with HNO_3 and H_2O_2, Cu concentration was determined by atomic absorption spectrometry (AAS) with graphite furnace (Perkin Elmer; SIMMA 6100). A calibration standard curve was prepared using dilutions of Cu (J.T. Baker), and the sample values were normalized to the values of DW.

RNA extraction. Total RNA was isolated from shoot and roots tissues as described by Logemann et al. [15]. Quality and quantity of RNA was checked visually prior to, and after DNAase digestion (TURBO DNAasa-free, Ambion) by denaturing gel electrophoresis and by photometric analysis ($A_{260/280}$ ratio). Absence of genomic DNA was confirmed by PCR, using primers designed from the intron sequence of At5g65080 gene [16].

Quantitative real-time RT-PCR. Total RNA (1 μg) was used as a template for reverse transcription reactions to synthesize single-stranded cDNA, using MMVL-RT reverse transcriptase (Promega) and an oligo(dT) primer (Promega), according to standard procedures. Gene-specific primers sets (detailed in Table 1) were designed by Primer3plus to amplify 70 and 150 bp in length. Real-time RT-PCR reactions were performed in a LightCycler system (Roche) using SYBR Green to monitor cDNA amplification. Equal amounts of cDNA, corresponding to a 1/12 dilution of the cDNA were used in each reaction, which contained 5 μL of Platinum SYBR Green I SuperMix-UDG (Invitrogen) 0.5 μL of BSA 20× (Invitrogen) and 5.0 pmol of forward and reverse primers in a total volume of 10 μL. The following standard thermal profile was used: 2 min at $50\,°C$, 2 min at $95\,°C$, 40 repeats of 5 s at $95\,°C$ and 15 s at $60\,°C$, and a final stage of 15 s at $72\,°C$. Data were analyzed using Light-Cycler Software (v3, Roche). Efficiency was determined for each sample and gene by LinRegPCR v7.5 using data obtained from exponential phase of each individual amplification plot as was described in [17]. Only those reactions were used for data evaluation for which transcripts were reliably detectable (Cp values below 35) and the reaction efficiency was above 1.5. Two technical replicates were done for each combination of cDNA and primer pair, and the quality of the PCR reactions was checked through analysis of the dissociation and amplification curves. The products were resolved by 3% agarose gel electrophoresis to confirm the DNA fragments of expected size. Transcript levels of genes were normalized to the respective transcript level of the expression values of *UBC* gene (At5g25760). Real-time PCR were performed in samples from at least three independent biological experiments and data were subjected to statistical analyses.

Measurement of root length. Plants (n = 40) were recorded with a Nikon Coolpix 5600 digital camera. For measurements of root length, pictures were analyzed by image analysis software Image

Table 1
Genes and gene-specific primers used for the real-time RT-PCR.

Gene	AGI (code)	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
COPT1	AT5G59030	GTTAATCCAAACCGCCGTGTA	GCCAGAGCGACGAGAAACAC
COPT2	AT3G46900	TTGGGGTAAGAACACGGAGGT	TGACACGTAGGATCGGTGAATG
COPT3	AT5G59040	CACCATCATCGTTCTTCCAACA	CGGCGAGACAGACCCAATAC
COPT4	AT2G37925	GGGATGTATGCACTCGCACTTA	GCTTATCGGCACCCTGTTTG
COPT5	AT5G20650	GAGAATCGCCGCATCCAAT	TGACGCCGAAAAGAAGAACC
ZIP2	AT5G59520	ACGTTGCGGTTAACCATCTC	CGAGGAAGACGGCAATAAAC
ZIP4	AT1G10970	TCACCATAGGCATAGTCACTC	AATCCCGAGCTCCAATATCTG
COX17-1	AT3G15352	GATTGATTCCTCCACCCACTTC	CATATCCTCTTCTTTGGTTTCGTC
COX17-2	AT1G53030	AGACAGTGCGTGTTCTTTGGAC	AAGCGGATTCACCGTGTTCTA
CCH	AT3G56240	CGTTGTCCTCAAAGTTGGTATGTC	CCTTTCACTGTCACCTTTTGCTC
ATX1	AT1G66240	TGTTCCAAGCCGTATCCTATCA	TCCACGCCTTCCATTTTCC
CCS1	AT1G12520	GCAAACTGGTCGAAAAGCTC	GTCAGGGCCTTTGAATTCTG
AT3G08950	AT3G08950	TTGGTTTCACTCATTGTCCCG	TTTGTCAATGGCAGCAGCTAAC
AT1G02410	AT1G02410	GGCATTCAGAATCTGGTACCGT	GCATCCCATCTGCAACATCA
HMA1	AT4G37270	GAGATTGTGCCTGTAGATTGCG	TCATTCCATGCCTTTGTAGCC
HMA5	AT1G63440	GGGAAGCCCGTTGTTGTGA	CCTTTGCTAACGGATGCTCACT
HMA6/PAA1	AT4G33520	AAGATACTGGAAAGCCAACCTCA	GCAAGCGTCTCGCCTAAACT
HMA7/RAN1	AT5G44790	TTACCCAGACGAATGGCTTC	TTGCCACCATAACAGCAGTC
HMA8/PAA2	AT5G21930	GCTTTGTGCTCCTTGGTCGT	CAGAATCCACTGGGGTGTTATTG
CDS1	AT1G08830	CCCTGAGGATGCTAATCGACAT	TGGCAATCAGTGATTGTGAAGG
FSD1	AT4G25100	TCGGCTCTTTCCCATTGCTT	TGGTCTTCGGTTCTGGAAGTCA
UBC	AT5G25760	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC

Tool 3.0 (http://ddsdx.uthscsa.edu/dig/itdesc.html). Data represent the mean of two independent experiments ± ES. Mann–Whitney test ($p < 0.05$) was used to test differences in root length.

Statistical analysis. We applied one way ANOVA and a Tukey *a posteriori* test to evaluate statistical differences among treatments. $p < 0.05$ was considered statistically significant.

Results and discussion

Our aim was to compare gene expression responses in adult plants under conditions of moderate deficit or excess of Cu, thus avoiding stresses as a result of either critical deficiency or chronic toxicity. First, we established an experimental set up that allowed us to ensure changes in Cu concentration of plants under two regimes of metal exposition. In doing so, we performed time-course experiments in adult plants exposed to Cu sulfate (50 μM) or BCS (50 μM) a Cu-specific chelator. Cu content was assessed by AAS in roots and shoots, and the results are shown in Fig. 1. After 24 h of Cu supply a remarkable increment of Cu content from 46.7 ± 4.3 to 512 ± 62.4 μg/g DW was detected in the roots of plants treated with this metal. In these plants, the increment of Cu in shoots varies from 7.2 ± 0.5 to 25.2 ± 1.9 μg/g DW after 24 h of Cu exposure. Even though, both shoots and roots showed a sustained accumulation of Cu over time, no further increment in the copper concentration was detected after longer periods (48 and 72 h) of treatment (data not shown). These data also confirmed that foliar Cu did not exceed 30 μg/g DW, a concentration that is a critical toxicity level of Cu in the leaves [18]. After 5 days of treatment with BCS we did not detect a decrease in Cu content in roots, however in shoots Cu content significantly decreased from 7.2 ± 0.5 to 3.4 ± 0.4 μg/g DW, a value that is below the critical level of 5 μg/g DW, which reflects a condition of Cu deficiency in plants [18].

In all experiments, we did not notice any visible signs of chlorosis or size variation in the shoots as a result of Cu or BCS treatments at the time of sample collection (Fig. 2A). However, in the case of plants exposed to BCS (Fig. 2B), we observed that they exhibited longer roots (13.2 ± 0.28 cm) when compared to control plants (9.62 ± 0.23 cm) (Fig. 2C). The observed phenotype is similar to that showed by plants transformed with an antisense sequence of *COPT1* a homologous of *CTR1* of yeast [6]. *COPT1* antisense transgenic plants displayed an increment of root length which was correlated with a decrease in Cu uptake. Thus, in our experiments the phenotype of BCS-treated plants is consistent with that described
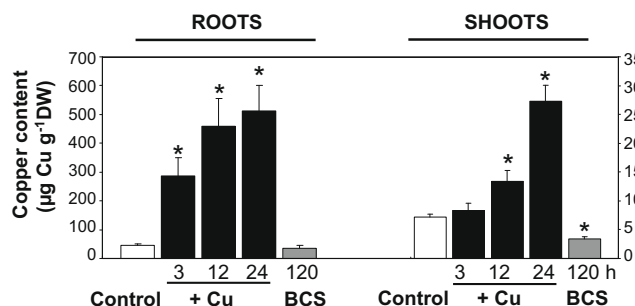
CONTROL   **+Cu**   **BCS**

Fig. 2. Hydroponically grown plants under two regimen of copper. (A) Shoots of 5-week-old plants exposed for 24 h to copper (+Cu) or 5 days to BCS prior sampling. (B) Roots of plants exposed for 5 days to BCS. (C) Measurement of root length, data represent the mean of two independent experiments ± ES (Mann–Whitney test, $p < 0.05$).

in Cu-deficient conditions, indicating that roots were sensing low copper levels, even though they manage to maintain stable levels of copper content. Taken together, these results suggest that under conditions of copper exposure shoots and roots were able to regulate their copper content to maintain stable levels of the metal, whereas a condition of Cu-induced deficiency elicited a differential response in the two tissues. We did not find significant changes in the contents of Fe and Zn in BCS-treated plants indicating that Cu deficiency did not affect the accumulation of other essential elements (data not shown).

Changes in the expression levels of genes *FSD1* (encoding FeSOD) and *CSD1* (encoding CuZnSOD) have been reported as good indicators of the plant response to Cu deficiency [7,19] and Cu excess respectively [7,20]. Therefore, we examined the expression levels of these marker genes using real-time RT-PCR to assess whether the variations in Cu contents attained with our treatments were able to produce a transcriptional response in the plants. The results indicated that the expression of *CSD1* in shoots and roots increased during the initial 12 h of Cu treatment, reaching its maximum of expression at 24 h, whereas *FSD1* was significantly down-regulated during the same period of time (Fig. 3). After copper depletion, real-time RT-PCR analysis revealed that *CSD1* was significantly down-regulated in shoots, while *FSD1* was up-regulated (Fig. 3). Our data showed a good a correlation between copper content and *CSD1* and *FSD1* transcript levels in both tissues. Thus, these results are in agreement with the gene expression patterns observed for both genes under similar conditions in arabidopsis and other species of plants [7,19].

Once the experimental conditions for Cu and BCS treatments were established, we examine the transcriptional response of 18 genes involved in Cu homeostasis (Fig. 4) in order to obtain additional insights into the mechanisms that underlie the adaptive response of adult plants to fluctuations in Cu availability. The genes were clustered according to its functional role in Cu metabolism, the expression of each gene was calculated relative to *UBC* expression and gene expression data is shown considering 100% the highest level measured in each tissue in order to facilitate its comparison. Five members belonging to COPT family, which are in-

Fig. 1. Copper status in *A. thaliana* plants exposed to Cu-excess or Cu-deficient conditions. Cu accumulation was determined in shoots and roots of 5-week-old plants grown in hydroponic medium. For Cu-excess or Cu-deficient treatments, plants were transferred to medium supplemented with 50 μM copper (+Cu) for 24 h or with 50 μM bathocuprein (BCS) for 5 days. Control plants were transferred to fresh medium at the onset of the respective treatments and they were harvested at the same time as treated plants. Copper contents were assessed by AAS in plants at various time points. Values are given as means ± the standard error of three biological replicates and the asterisks indicate significant differences between control and treated plants (ANOVA, $p < 0.05$).
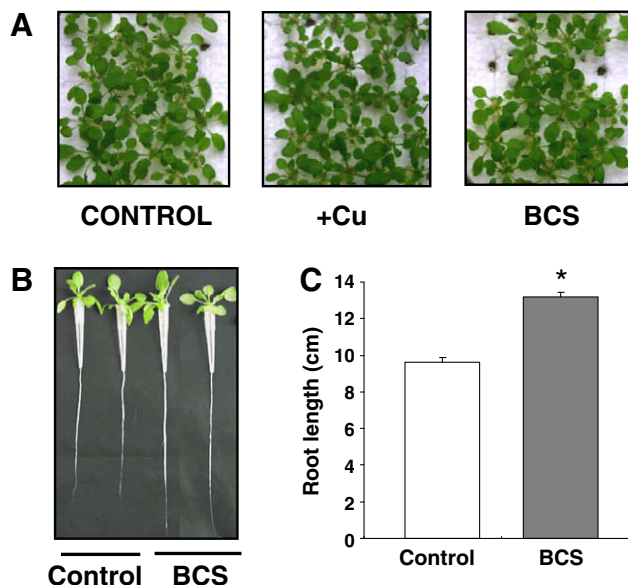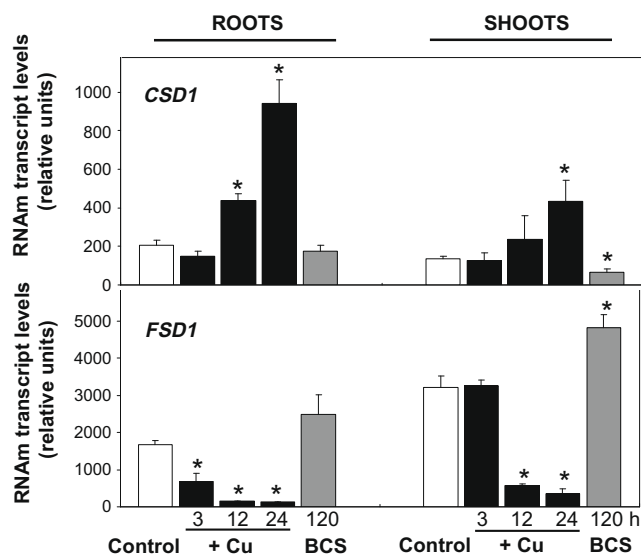
Fig. 3. Transcript levels of *FSD1* and *CSD1* genes in plants exposed to copper excess or deficiency. Transcript levels were determined in the shoots and roots of 5-week-old plants. Plants were growth in hydroponic medium and then transfer to the same medium supplemented with 50 μM copper (+Cu) for 24 h or with 50 μM BCS for 5 days. Control plants were transferred to fresh medium at the onset of the respective treatments and they were harvested at the same time as treated plants. For each gene transcript level was normalized toward *UBC*. Values are given as means ± the standard error of three biological replicates and the asterisks indicate significant differences between control and treated plants (ANOVA, $p < 0.05$).

Fig. 4. Regulation of mRNA levels of copper homeostasis genes by copper availability. Transcript levels of 18 genes were determined by quantitative real-time RT-PCR in excised shoot (S) and roots (R) of hydroponically cultivated in plants treated for 24 h with 50 μM copper sulfate or 5 days with 50 μM BCS. For each gene transcript level was normalized toward *UBC* in the corresponding samples. Values are mean relative transcript levels calculated from two technical replicates from a total of three independent experiments. The results are presented as a percentage of the highest value of relative transcript level.

volved in Cu uptake transport were analyzed. Our results indicated that comparing to controls plants, transcript levels of *COPT1*, *2*, and *4* were significantly down-regulated in roots, whereas *COPT2* and *4* were down-regulated in shoots in response to Cu excess. For *COPT3* we did not obtain reliable real-time RT-PCR results (Cp > 35 cycles), since this gene exhibited very low level of expression in these tissues [21]. Regarding *ZIP2* and *ZIP4*, which are member of a gene family encoding transporters of divalent metal, including Zn and Cu [22,23], our results indicated that *ZIP2* was significantly down-regulated in Cu excess in both tissues while under limited copper condition their expression was up-regulated in shoots. These data are in agreement with a previous report [23] showing that *ZIP2* in plants of *A. thaliana* was induced in Cu deficiency and repressed in Cu excess. *ZIP4*, on the other hand, was up-regulated in response to Cu excess in both tissues, but we did not detect any significant change in BCS-treated plants. Thus our data support the idea that *ZIP2* and *COPT2* might be useful to monitor copper status in arabidopsis.

The analysis of the expression of genes encoding for Cu chaperones revealed a similar patterns of regulation by Cu excess, thus *ATX1*, *CCS*, *COX17-1* were up-regulated under excess of copper in both tissues (Fig. 4). In addition, we included in our analysis two putative mitochondrial metallochaperones (At3g08950 and At1g02410) which were significant up-regulated just in shoots. These two genes exhibit sequence similarity with Sco1p and Cox11p from yeast, which are involve in copper delivery to cytochrome *c* oxidase [24,25]. *CCH* was the only chaperone that showed a reduced expression level under conditions of Cu excess. Furthermore, the response of *CCH* to variation in copper contents followed an expression pattern similar to that of *COPT2*, *ZIP2* and *FSD1*. Interestingly, bioinformatic analysis of non-coding regions or regulatory regions of these four genes revealed the presence of several repetitions of a GTACT motif, which in turn has been suggested to be responsible for the Cu-mediated transcriptional suppression of FeSOD in moss and higher plants [26]. Therefore, transcriptional behavior of *COPT2*, *ZIP2*, and *CCH* in response to Cu seems to be
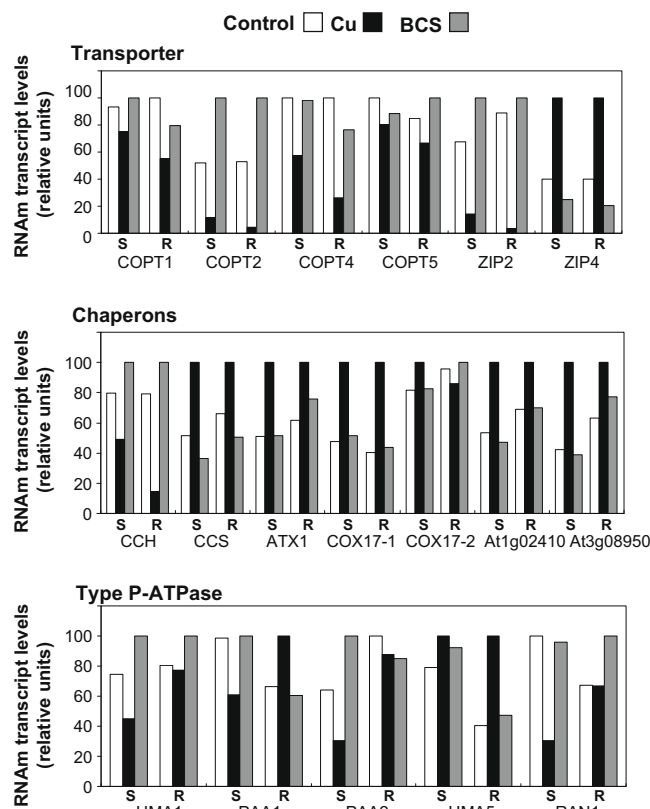
in agreement with the idea of a coordinated regulation through a common transcription factor. Recently, it has been shown that the transcription factor *SPL7* of arabidosis bind directly to GTAC motifs and in absence of *SPL7* the response to low copper was abolished in these four genes [27].

Finally, the P-type ATPases *HMA1*, *PAA1*, and *PAA2*, which are required for Cu delivery into the chloroplast, were down-regulated in shoots of plants exposed to copper (Fig. 4), probably as part of a mechanism to reduce copper uptake and preserve thylakoids function. On the contrary, in Cu deficiency *HMA1* and *PAA2* were up-regulated highlighting the need to maintain a proper Cu supply to chloroplasts. Regarding *HMA5* expression, its induction in roots (Fig. 4) might result in an increment of copper efflux, supporting a role of *HMA5* in copper detoxification [28,29]. Transcriptional regulation of P-type ATPases involved in copper efflux has been reported principally in bacteria [30] while in mammalian its function is regulated by post-translational mechanism including redistribution of the protein from the Golgi complex to plasma membrane [31].

In summary, our results showed that members of three functional categories of proteins involved in copper homeostasis: transporters, chaperones and P-type ATPases changed its expression levels in response to copper availability. In arabidopsis the transcriptional behavior of these genes followed a pattern that is characteristic of genes involved in micronutrient homeostasis, i.e. genes encoding proteins involve in micronutrient uptake are up-regulated in response to deficit and down-regulated in excess.

Despite the remarkable conservation among components of Cu homeostasis in yeast and higher eukaryotes, several studies indicate that the transcriptional regulation of genes encoding proteins involved in Cu homeostasis (uptake, intracellular distribution and efflux) does not represent a relevant mechanism to control intracellular levels of Cu in mammalian [32,33]. In contrast to that observed in mammalian models, our results indicate that transcriptional regulation plays an important role in Cu homeostasis in plants, shedding some light on the potential components involved in the early adaptive response to Cu. Considering that plants are sessile organisms, transcriptional regulation of components involve in copper homeostasis to reinforce the capacity to adapt to the wide range of environmental Cu availability.

Acknowledgments

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