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Comparison of global responses to mild deficiency and excess copper levels in *Arabidopsis* seedlings†

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Copper is an essential micronutrient in higher plants, but it is toxic in excess. The fine adjustments required to fit copper nutritional demands for optimal growth are illustrated by the diverse, severe symptoms resulting from copper deficiency and excess. Here, a differential transcriptomic analysis was done between *Arabidopsis thaliana* plants suffering from mild copper deficiency and those with a slight copper excess. The effects on the genes encoding cuproproteins or copper homeostasis factors were included in a CuAt database, which was organised to collect additional information and connections to other databases. The categories overrepresented under copper deficiency and copper excess conditions are discussed. Different members of the categories overrepresented under copper deficiency conditions were both dependent and independent of the general copper deficiency transcriptional regulator SPL7. The putative regulatory elements in the promoter of the copper deficiency overrepresented genes, particularly of the iron superoxide dismutase gene *FSD1*, were also analysed. A 65 base pair promoter fragment, with at least three GTAC sequences, was found to be not only characteristic of them all, but was responsible for most of the *FSD1* copper-dependent regulations. Moreover, a new molecular marker for the slight excess copper nutritional status is proposed. Taken together, these data further contribute to characterise copper nutritional responses in higher plants.

Introduction

The reiterative use of agricultural copper (Cu) treatments enhances environmental levels of Cu. Cu has been used as a pest protectant for cereal crops since the eighteenth century[1] and, nowadays, it is one of the few anti-microbial treatments allowed in organic farming.[2] However, given Cu phytotoxicity[1,3,4] and the persistence of environmental Cu,[5] the use of Cu in crops has to be optimised.[6] In fact, the European Union has already voiced some concerns about the abuse of Cu-based crop treatments.[7] Deficiency or low Cu bioavailability in soil also affects plant productivity, causes major economic losses and reduces the nutritional value of crops, thus affecting human food. Cu deficiency alters the functioning of electron transport chains in both mitochondria and chloroplasts through the malfunctioning of cuproproteins such as cytochrome *c* oxidase and plastocyanin. Plants, as well as other eukaryotes, have developed sophisticated mechanisms to tightly control Cu homeostasis in response to changing environmental Cu bioavailability. Recent studies with *Arabidopsis thaliana* (*A. thaliana*) have allowed the characterisation of the diverse families and components involved in plant metal acquisition, distribution and tolerance.[8,9] Furthermore, gene expression analyses now decipher coordinated mechanisms of regulation and response to Cu limitation.[10,11] With the exception of transcriptional regulators, all the components of Cu homeostasis networks

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seem to be conserved in eukaryotes.[12] Briefly, Cu uptake into the cytosol in eukaryotes takes place through a family of cell-surface high affinity Cu transport proteins, denoted CTR and COPT, in yeast/animals and plants, respectively. In *A. thaliana*, different results suggest that COPT1 and COPT2 are plasma membrane proteins, whereas COPT3 and COPT5 proteins function in intracellular Cu distribution.[13] Under conditions of low Cu availability, the expression of plasma membrane metal permeases COPT1 and COPT2, which mediate high affinity Cu transport towards the cytosol, is activated.[14] COPT1 participates in Cu uptake from soil through the root apex.[15] Moreover, COPT5 is involved in the mobilisation of Cu from the lumen of the vacuole or prevacuolar compartments towards the cytosol in response to extreme Cu-deficient conditions.[16,17] Other components in the Cu homeostasis network are soluble cytosolic cuprochaperones,[18] one of their tasks is to provide Cu to cytosolic targets, such as the CCS cuprochaperone, which delivers Cu to the Cu and zinc (Zn) superoxide dismutase (Cu/ZnSOD). ATX1 is another cuprochaperone that delivers Cu to the P-type ATPases of Cu^+ (Cu^+-ATPases) at the endoplasmic reticulum to pump Cu into the lumen, where it is loaded into cuproproteins in the secretory pathway. *Arabidopsis thaliana* has two homologues of this cuprochaperone: ATX1 and CCH.[8] Based on the presence of conserved Cu-binding motifs,[19] it has been proposed that more than 200 proteins encoded by the *A. thaliana* genome bind Cu. The function of only a small fraction of them is already known, including plastocyanin, cytochrome *c* oxidase, ethylene receptors, multicopper oxidases (MCO), the laccases involved in the synthesis of extracellular matrix components, and MCO, LPR1 and LPR2 related to the perception of phosphate starvation responses.[20]

The transcriptional response to Cu deficiency in *A. thaliana* is mediated by one member of the Zn finger transcription factor family, namely SQUAMOSA-promoter binding-like protein (SPL). SPL7 is essential for responses to Cu deficiency *in vivo* through its binding to the GTAC motifs in the promoter of target genes.[10,11] Although the mechanism remains currently unknown, the SPL7 repression in the presence of Cu can be mediated by the displacement of Zn by Cu from the Zn fingers of the transcription factor, as recently described *in vitro* for the *Chlamydomonas reinhardtii* orthologue.[21] One of the several SPL7-mediated strategies used by *A. thaliana* when Cu is limited is to replace non-essential cuproproteins with other metalloproteins, usually iron (Fe) proteins, that carry out a similar role, probably to save Cu for essential cuproproteins such as plastocyanin.[22] Substitution of Cu/ZnSOD for FeSOD under Cu-limited conditions is carried out by the SPL7 transcription factor, which activates the expression of *FSD1* (encoding chloroplastic FeSOD) and promotes the degradation of *Cu/ZnSOD* mRNAs through the induction of microRNA *miR398*.[23] Other Cu-regulated miRNAs include *miR397*, *miR408* and *miR857*, which are involved in the degradation of the mRNAs encoding cuproproteins such as plantacyanins and laccases.[23,24] Therefore, regulation by miRNAs is a widespread response to Cu deficiency, as is the case for other nutrients.[20]

Although large-scale gene expression analyses under Cu deficiency conditions have been recently reported,[10,11] genome-wide changes affecting plants under mild Cu excess conditions have not yet been characterised. Furthermore, the use of molecular markers for the Cu nutritional status is important in crops in order to establish the endogenous metal content perceived by plants and to prevent the deleterious toxicological effects deriving from excess Cu supply. A marker for mild Cu stress would be of interest to adjust the appropriate Cu dose in crop treatments and to anticipate any reduction in the physiological parameters leading to diminished plant production if excess Cu supply continues. However, most gene expression analyses under Cu excess conditions have been carried out at high Cu concentrations (25–50 μM), where reduced growth and other physiological symptoms are already evident.[25–29] The main objectives of the current work are to define Cu sufficiency limits at the molecular level and to provide new molecular markers for mild Cu deficiency and excess conditions. For these purposes, global changes in the gene expression under mild Cu deficiency and Cu excess conditions were compared. Information on Cu homeostasis factors was compiled in a CuAt database (www.uv.es/cuatlab/cuatdb). Moreover, the analysis of a *cis* regulatory element in *FSD1*, one of the best overrepresented genes under conditions of Cu deficiency *vs.* excess, is presented. The results shown herein underscore the differential regulation of the Cu homeostasis network and several general cellular processes, further contributing to understand plant responses to a mild Cu-unbalanced supply.

Results and discussion

Metal content under conditions of different Cu availabilities

In order to evaluate endogenous metal content in accordance with the Cu present in the medium, we grew *A. thaliana* seedlings for 6 days on homemade MS plates[30] with Cu concentrations ranging from 0 to 50 μM. In addition, commercial MS (Sigma) plates with and without the Cu chelator bathocuproinedisulphonic acid (BCS) at 100 μM were also included (Fig. 1). As expected, endogenous Cu concentrations increased as Cu was added to the medium. According to the sufficiency range (5–20 μg per gram of dry weight),[31] the Cu concentrations in the medium below 0.5 μM represented suboptimal Cu availability for plants, whereas those in the medium above 5 μM Cu constituted excess for the seedlings grown on plates (Fig. 1). We should note that standard commercial MS medium, widely utilised by the *A. thaliana* community often as the control condition, is included within the mild Cu deficiency range (2.13 ± 1.07 μg Cu per gram of dry weight), a fact also indicated by other laboratories.[10,22] The levels of the two other metals participating in the SOD substitution process in plants, Zn and Fe, were also measured (Fig. 1). Both metals remained at the sufficiency levels within the whole Cu concentration range used in the experiment. Zn concentration slightly, but significantly, increased within the Cu excess range, whereas Fe content slightly increased as Cu increased within the Cu deficiency and sufficiency range, but decreased under Cu excess conditions (Fig. 1).

No differences in symptoms were observed in the wild-type (WT) plants grown within the Cu 0 to 10 μM range. Thus, root growth, a sensitive parameter to Cu levels, remained unaltered

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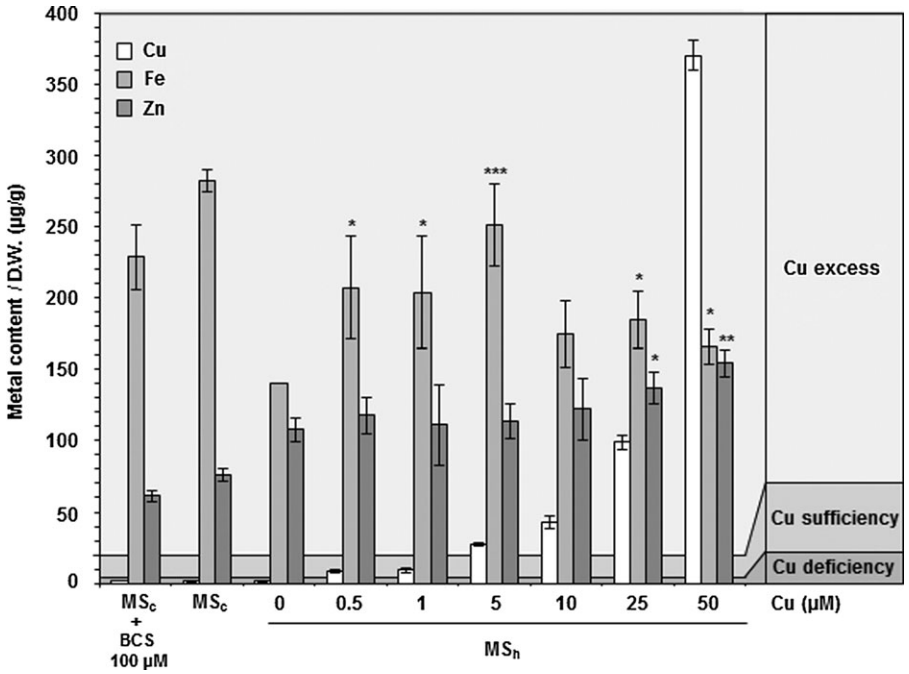

Fig. 1 Endogenous metal content and Cu status in the *Arabidopsis* seedlings grown on different Cu-supplemented media. The Cu (white bars), Fe (light grey bars) and Zn (dark grey bars) contents from 6-day-old wild-type (WT) seedlings grown on MS media (commercial, MS_c; homemade, MS_h) supplemented with BCS or Cu, as indicated. Values are means ± SD of three biological replicates. DW, dry weight. Asterisks, indicated in the Fe and Zn contents, represent significant differences in relation to the Cu 0 µM medium (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$). The deficiency, sufficiency and excess ranges are indicated.[31]

within this Cu concentration range, and up to at least 20 µM Cu (Fig. S1a, ESI†). Other parameters, such as lipid peroxidation (measured as malondialdehyde, MDA, content) and chlorophyll content, showed no significant changes at Cu concentrations below 25 µM (Fig. S1b and c, ESI†). Moreover, the *A. thaliana* plants grown on soil under either Cu deficiency (no added Cu) or Cu excess (100 µM Cu) conditions showed apparently similar visual symptoms when compared to sufficiency (2.5 µM Cu), such as leaf chlorosis, reduced size and fertility (Fig. S2, ESI†), hindering the Cu nutritional status diagnosis.

Gene expression changes under conditions of different Cu availabilities

In order to better characterise the higher plant responses at the molecular level to the sub- and supra-optimal Cu levels in the growth medium, a global profiling analysis of gene expression under limiting or exceeding Cu conditions was performed. We chose pre-symptomatic Cu concentrations. Hence changes in gene expression represent adaptive responses rather than secondary growth or toxicity effects. In addition, our analyses present other novelties and advantages, such as the minimised contribution of general stress responses since both conditions represent mild stress, thus highlighting specific mild Cu deficiency or excess responses. To this end, *A. thaliana* seedlings were grown under a long day photoperiod for 6 days on commercial MS plates to be compared with the seedlings grown in the same medium supplemented with 10 µM Cu. Hereafter, these two Cu status conditions are, respectively, denoted low Cu and high Cu since the endogenous Cu levels in MS were below the optimal supply, but were above the excess threshold at 10 µM Cu (Fig. 1).[31] In order to validate the microarray conditions for low and high Cu, we determined the expression pattern of the genes previously described to be Cu-responsive by semi-quantitative PCR (sqPCR) (Fig. S3, ESI†). The gene expression changes expected in the Cu homeostasis factors in response to low Cu include the aforementioned SOD substitution. While *FSD1* had a lower expression under high Cu conditions as compared to low Cu conditions, the expression of the genes encoding both cytosolic and chloroplastic Cu/ZnSODs (*CSD1* and *CSD2*, respectively) was higher (Fig. S3, ESI†), as previously reported.[22] Furthermore, the expression of transporters such as *ZIP2* (ZRT, IRT-like protein 2)[32] and *COPT2* (ref. 13) was lower under high Cu conditions as compared to low Cu conditions, while that of *HMA5*, a P-type ATPase that plays a role in Cu efflux,[33] was higher. Metallochaperones responded differentially to low Cu levels in accordance to their function. For example, *CCH* (copper chaperone), a plant-specific chaperone,[34] was overrepresented under low *vs.* high Cu conditions, whereas under our experimental conditions, *CCS* (Cu chaperone for Cu/ZnSOD and the only chaperone for both cytosolic and chloroplastic Cu/ZnSODs)[35,36] was overrepresented under high *vs.* low Cu conditions (Fig. S3, ESI†).

For the microarray expression analysis, samples were compared as high *vs.* low Cu. A median $\log_2$ ratio of 1 (2-fold difference in expression) was used as a cut-off criterion. Of the 232 differentially expressed genes identified, 125 were overrepresented under high Cu conditions (ratio >1) and 107 were overrepresented under low Cu conditions (ratio <−1). The biological processes affected by Cu status were identified with the Gene Ontology (GO) annotation,[37] performed with the

Table 1 Functional categories overrepresented under low and high Cu conditions in *Arabidopsis* seedlings. Summary of the microarray analysis results from the 6-day-old wild-type (WT) seedlings grown on commercial MS media without (Low Cu) and with Cu 10 μM (High Cu)

Low Cu			High Cu		
Functional category	No. of genes	Marker gene	Functional category	No. of genes	Marker gene
Ion homeostasis	10	*COPT2*	Ion homeostasis	2	*CCS*
Responses to stress	17	*FSD1* *CYP706A1*	Responses to stress	19	*CSD1* *SDH1-2* *RbohI*
Sulphur metabolism	13	*BUS1*	DNA modification	12	*HDA18*
Cell growth	18	*GDPD6* *EXPB3*	Cell division	18	*BUB3.2*
Signal transduction	22	*miR398b* *CAS* *ACA1*	Signal transduction	17	*PRR5* *GRP7*

FatiScan (http://fatiscan.bioinfo.cipf.es/) and GeneCodis2.0 (http://genecodis.dacya.ucm.es/) programmes (Table 1). All the differentially expressed genes in the microarray are shown in Tables S1 and S2 (ESI†). The different genes involved in ion homeostasis, responses to stress, and signalling processes were overrepresented under each Cu condition. Yet DNA modification and cell division genes were overrepresented under high *vs.* low Cu conditions (Table S1, ESI†), and sulphur metabolism and cell growth genes were overrepresented under low *vs.* high Cu conditions (Table S2, ESI†).

Establishment of a CuAt database

The first category selected to be analysed in this work was ion homeostasis as it has been a main focal point in our laboratory in recent years. Certain genes participating in Cu homeostasis were overrepresented in this group (Table 1; Tables S1 and S2, ESI†). In order to classify the Cu homeostasis-related factors and to facilitate the search for information about *A. thaliana* Cu homeostasis components, we created a database which we called the CuAt database (Table S3, ESI†). This compilation includes the proteins that bind Cu,[19,38] and it is connected to other databases and websites (TAIR; *Arabidopsis* eFP Browser; ENSEMBL; KEGG; and PUBMED). Transcriptomic data, such as the microarray value under the different Cu status analysed in this work and the number of putative regulatory GTAC motifs in the 500 base pair (bp) promoters of the genes encoding Cu-related proteins, are also included. The CuAt database is accessible to the public on our laboratory web site (www.uv.es/cuatlab) and it is modified as new relevant information on Cu homeostasis emerges. Table 2 summarises the seven families into which the CuAt database has been organised, along with the corresponding subgroups. The main families included in the CuAt database are transporters, chaperones, chelators, target cuproproteins and regulatory factors. We also included two other categories, these being miscellaneous components (others) and other Cu-related proteins, which remain unclassified to date (Table 2 and Table S3, ESI†). The number of members already related to Cu homeostasis in a subgroup in terms of total number of members (shown in brackets) is also indicated, along with the number of genes overrepresented at low or high Cu levels in our microarray analysis (Table 2).

Table 2 CuAt database families. Summary of the number of Cu homeostasis-related genes arranged into families. The number of overrepresented members at either low or high Cu levels in the microarray analysis is also indicated for each subgroup. The total number of members for each subgroup is given in parentheses. The complete dataset is shown in Table S3, ESI

		Microarray	
Family	Genome	Low Cu	High Cu
1. Cu Transporters			
1.1. COPT-type transporters	6	1	
1.2. Cu ATPases	4 (8)		
1.3. Others			
1.3.1. ZIPs	2 (12)	1	
1.3.2. Cu chelators transporters	2 (9)	1 (1)	
1.3.3. Others	1		
2. Cuprochaperones	45	1	1
3. Cu chelators			
3.1. Metallothioneins	8 (9)	1	(1)
3.2. Others	5	2	
4. Targets			
4.1. Ethylene receptor	1		
4.2. Cu/Zn superoxide dismutases	3		2
4.3. Blue copper proteins or cuprodoxins			
4.3.1. Plastocyanin	2		
4.3.2. Plantacyanin	1		1
4.3.3. Others	44 (45)		
4.4. Cytochrome *c* oxidase	31 (85)	1	
4.5. Diamine oxidase	17 (26)		
4.7. Blue oxidases or multicopper oxidases (MCOS)			
4.7.1. Ascorbate oxidases	11 (14)		
4.7.2. Laccases	17		
4.7.3. Others	22 (25)		
5. Regulators			
5.1. SPLs	3 (17)		
5.2. miRNAs	7		
6. Others			
6.1. Reductases	(8)		
6.2. Mutants	2		
7. Unclassified	284	5	4

Analysis by real-time quantitative PCR of the representative members of the Cu-regulated categories

Representative members of each category that were overrepresented under low and high Cu conditions in our microarray analysis were selected from among the higher microarray values (Table 1) to determine their expression levels by real-time quantitative PCR (qPCR).

Global expression changes under high *vs.* low Cu (Table S1, ESI†) conditions included the Cu homeostasis factors that had

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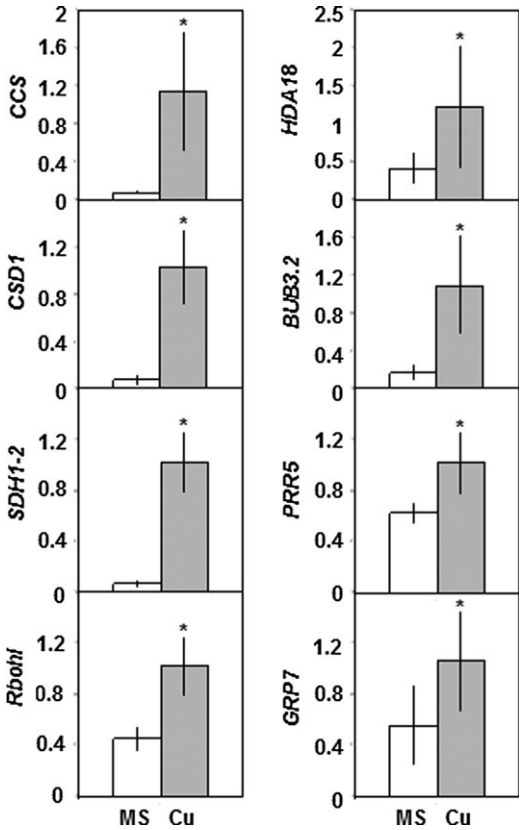

Fig. 2 Expression of the high Cu-representative genes in the *Arabidopsis* seedlings grown on different Cu-supplemented media. Relative expressions of the *CCS*, *CSD1*, *SDH1-2*, *RbohI*, *HDA18*, *BUB3.2*, *PRR5* and *GRP7* genes. The total RNA from the 6-day-old WT seedlings grown on commercial MS medium without (MS; white bars) and with Cu 10 μM (Cu; grey bars) was isolated and retrotranscribed to cDNA. The mRNA amounts of each gene were measured by qPCR with specific primers and were normalised to the *UBQ10* expression. mRNA levels are expressed in relative units to the high Cu conditions. Values are the means ± SD of four biological replicates. Asterisks represent significant differences ($P < 0.05$).

already been reported to respond to Cu status. In this sense, the *CCS* expression was used as a control in qPCR for high Cu overrepresented genes (Fig. 2). According to the oxidative stress that Cu has been proposed to induce, one of the best representatives in this category was *CSD1*, which was already reported to respond to Cu status, and was confirmed by qPCR under our conditions. Moreover, the expression of a nuclear gene encoding the flavoprotein subunit of succinate dehydrogenase, *SDH1-2*,[39] was also checked and confirmed (Fig. 2), indicating the mitochondrial involvement in the perception/signalling of these oxidative stress conditions. Furthermore, the expression of the guard cells and root xylem NADPH respiratory burst oxidase *RbohI*[40] also increased under high *vs.* low Cu conditions (Fig. 2), suggesting a deliberate generation of reactive oxygen species (ROS) as part of the Cu signalling process. In the DNA modification and cell division categories, the genes encoding histone deacetylase 18 (*HDA18*) and *BUB3.2* were the candidates chosen to check their expressions by qPCR (Fig. 2). *BUB3.2* encodes a homologue of the yeast and human BUB3 (BUDDING UNINHIBITED BY BENZYMIDAZOL 3) protein, which functions in the spindle assembly checkpoint control.[41]

It is noteworthy that while morning members of the circadian clock, *e.g.*, *LHY* and *CCA1*, are overrepresented under Cu deficiency (Table S2, ESI†) conditions, as previously reported,[42] evening circadian clock components pseudoresponse regulator *PRR5* and glycine-rich RNA-binding protein *GRP7* (ref. 43 and 44) are among the best representatives in the signalling category under high Cu (Table S1, ESI†) conditions, whose expression was also corroborated by qPCR (Fig. 2). These results further reinforce the proposed interaction between Cu homeostasis and the circadian clock.[42]

Of the genes overrepresented under low Cu conditions (Table S2, ESI†), *SPL7* is the master transcriptional activator under Cu deficiency conditions.[10] *COPT2* and *FSD1* are among the best representative genes under low Cu conditions and their expression patterns are mostly dependent on SPL7.[10] We used the *FSD1* expression as a control of SPL7-dependent regulation by checking its expression in the *spl7* mutant at the different Cu levels (Fig. 3). The substitution of Fe SOD for the Cu–Zn SOD counterpart is a well-documented process which occurs under Cu-deficiency conditions and is mediated by the *miR398* expression targeting *CSD1* and *CSD2.*[23] It is worth mentioning that in addition to miR398, our data show other miRNAs (*miR165*, *miR169, miR319* and *miR395*) that target drought resistance, sulfate transport and circadian-related transcription factors,[45] which are overrepresented under Cu deficiency conditions (Table S2, ESI†).

COPT2 has been shown to be partially dependent on SPL7, as published elsewhere.[10] Among the most highly expressed genes in the stress category we find *CYP706A1*, which encodes a cytochrome P450 family member. In addition to the expression obtained in the microarray (Table S2, ESI†), this gene's Cu-regulated expression was corroborated by qPCR and was shown to be partially dependent on SPL7 (Fig. 3). Another member of the cytochrome P450 family, *CYP79F1*, also known as *BUS1* and related to the aliphatic glucosinolate biosynthetic pathway from methionine,[46] was selected in the sulphur biosynthesis category. *BUS1* was also partially SPL7-dependent (Fig. 3). In addition to *BUS1*, other genes specifically involved in this pathway (*e.g.*, *CYP83A1*, *MAM1*, *GSTF11* and *UGT74C1*) were overrepresented under Cu deficiency conditions (Table S2, ESI†). In the cell expansion category, glycerophosphodiester phosphodiesterase *GDPD6* and expansin *EXPB3* were seen to be among the most differentially expressed genes, and their expressions were confirmed by qPCR. Whereas the *GDPD6* expression was mostly independent of SPL7, the *EXPB3* expression was partially SPL7-dependent (Fig. 3). The expansin *EXPB3* expression was demonstrated to be repressed by the E2Ff transcription factor[47] which, accordingly, had a $\log_2$ ratio of +1.459 under our experimental conditions (Table S1, ESI†). Moreover, the other genes regulated by E2Ff, such as *EXPA8*, *UGT74C1* and *UGT78D2*, also obtained high values in our microarrays (Table S2, ESI†). Finally in the signalling category, *CAS* was one of the best representative genes, which encoded a chloroplast-localised protein, and is considered a primary

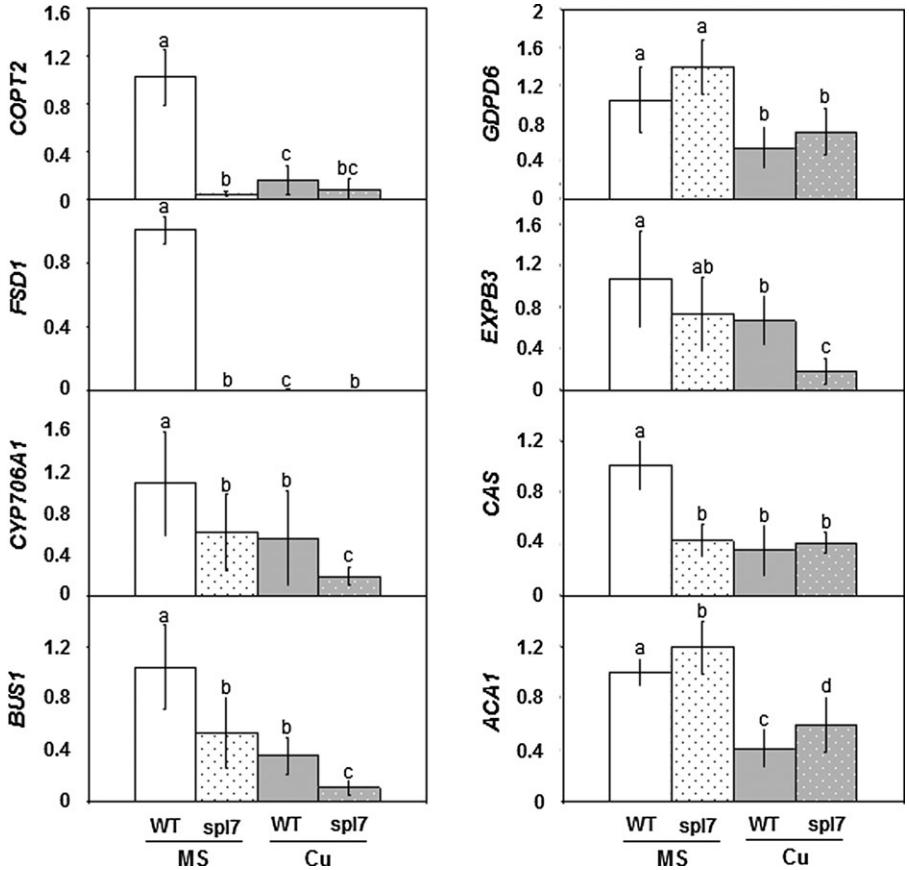

Fig. 3 Expression of the low Cu-representative genes in the *Arabidopsis* seedlings grown on different Cu-supplemented media. Relative expressions of the *COPT2*, *FSD1*, *CYP706A1*, *BUS1*, *GDPD6*, *EXPB3, CAS* and *ACA1* genes. The total RNA from the 6-day-old WT (plain bars) and the SPL7 knockout (spl7; dotted bars) seedlings grown on commercial MS medium without (Low Cu; white bars) and with Cu 10 μM (High Cu; grey bars) was isolated and retrotranscribed to cDNA. The mRNA amounts of each gene were measured by qPCR with specific primers and were normalised to the *UBQ10* expression. mRNA levels are expressed in relative units to WT seedlings under low Cu conditions. Values are the means ± SD of at least five biological replicates. The different letters above the bars represent significant differences ($P < 0.05$).

transducer of external Ca^{2+} in plants.[48] The increased *CAS* expression under low *vs.* high Cu conditions was SPL7-dependent (Fig. 3). Moreover, carbonic anhydrase *ACA1* was another calcium-related representative gene in this category, whose expression was SPL7-independent (Fig. 3).

Despite the differences with other global analysis studies, the *COPT2*, *FSD1*, *CYP706A1*, *CAS* and *ACA1* expressions in seedlings (Fig. 3) reproduce the pattern previously described when comparing adult (6-week-old) Columbia (Col 0) and *spl7-2* mutant plants in a recent whole transcriptome RNA-Seq analysis.[11]

The promoters of some of the most overrepresented genes under low Cu conditions exhibit three or more GTAC boxes in a promoter region shorter than 65 bp included in the 500 bp upstream of the gene transcriptional start (Table 3). Despite a single GTAC box being described as responsive to Cu deficiency SPL7-mediated transcriptional regulation,[10] a theoretical global study conducted on the 500 bp proximal promoters of our microarray genes indicated no correlation between the presence of a putative GTAC box and their Cu regulation (Table 3). Accordingly, the average number of expected GTAC boxes in 500 bp is 1.941, accounting for there being every likelihood of finding this short motif in relatively long sequences. However, recurrence of these boxes (≥3 GTAC boxes) in short regions (≤65 bp in the 500 bp upstream) is quite unlikely (1.977×10^{-3}). The genes with these characteristic promoter elements found in the current study were *FSD1*, *miR398b, ZIP2*, *COPT2*, *YSL2*, *CCH*, *LHY*, *CAS* and *SMC6B* (Table 4 and Fig. S4, ESI†), all of which were clearly overrepresented under Cu deficiency conditions (Table S2, ESI†). These findings enabled us to consider these GTAC repetitions as candidate *cis* elements, which are probably involved in transcriptional regulation under Cu deficiency conditions.

Table 3 Cu regulation and GTAC boxes in gene promoters. Summary of the number of genes with the indicated characteristics. The number of genes overrepresented at either low or high Cu levels in the microarray analysis is also indicated for each characteristic. The promoter regions of 500 bp upstream of the transcriptional start were used in this analysis. The ≤65 bp region is included in the 500 bp upstream of the gene transcriptional start

		Microarray		
	Genome	Total	Low Cu	High Cu
Total genes	33602	487	107	125
Genes with GTACs	18802	273	62	62
Genes with ≥3 GTACs in ≤65 pb	293	13	9	0

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Table 4 Genes exhibiting a putative Cu deficiency-responsive *cis* regulatory element. The number of GTAC boxes is indicated for a promoter region of 500 bp upstream of the transcriptional start. The considered *cis* element consists in ≥3 GTAC boxes in a region of ≤65 bp included in the 500 bp upstream of the gene transcriptional start. The ratio value is the median $\log_2$ ratio at the high *vs.* the low Cu levels in the microarray analysis

Gene annotation	Gene name	MIPS code	No GTACs	No GTACs in ≤65 bp	Ratio
Fe superoxide dismutase	*FSD1*	At4g25100	6	4	−3.834
Target CSD1, CSD2 and Cyt *c* oxidase members	*miR398b*	At5g14545	7	3	−3.516
Zn transporter	*ZIP2*	At5g59520	5	3	−3.243
High-affinity Cu transporter	*COPT2*	At3g46900	4	4	−2.860
Oligopeptide transporter (yellow stripe-like)	*YSL2*	At5g24380	4	3	−2.593
Cu chaperone	*CCH*	At3g56240	4	4	−1.819
Myb-related transcription factor	*LHY*	At1g01060	3	3	−1.594
Chloroplast-localised calcium sensor	*CAS*	At5g23060	5	3	−1.497
Component of the SMC5/6 complex	*SMC6B*	At5g61460	3	3	−1.183

In line with this, the *FSD1* gene, based on both its response to Cu deprivation and the presence of potential *cis* regulatory elements (its promoter has six GTAC boxes, four of which are included in ≤65 bp), is one of the best candidates as a marker gene of Cu deficiency (Fig. 3 and 4). In order to explore the regulation of the *FSD1* gene expression in Cu deficiency in greater detail, a chimeric construct was cloned, which included a fragment of 1193 nucleotides (nt) of its promoter containing the six GTAC boxes (*pFSD1*) fused with the *uidA* coding region for β-glucuronidase (GUS) from *Escherichia coli* (*pFSD1*::*GUS*) (Fig. S5, ESI†). To study the effect of its GTAC boxes on Cu-dependent transcription, a *FSD1* promoter fragment of 1128 nt, where the 65 nt region comprising the four GTAC boxes candidates to be involved in the Cu response was eliminated (*pFSD1**), was fused with *uidA* (*pFSD1*::GUS*) (Fig. S5, ESI†). Both constructs were sequenced to ensure integrity, cloned in the binary *pFP101* vector and used for *Agrobacterium*-mediated transformation by floral dipping.[49] A sqPCR analysis of the *GUS* and *FSD1* expressions in both transgenic seedling types grown on the described low and high Cu media was done (Fig. 4a). Whereas *GUS* behaved similarly to the endogenous *FSD1* gene in terms of Cu deficiency responsiveness in the *pFSD1::GUS* plants, *GUS* induction was lost mostly in the plants from which the four GTAC boxes were eliminated (*pFSD1*::GUS*). In order to quantify this effect, qPCR was performed for the *GUS* transcript. As shown in Fig. 4b, most of the induction (above 99.9%) was lost when the four GTAC boxes were eliminated from two independent transgenic lines, indicating the importance of this element (≥3 GTAC boxes in ≤65 bp in the 500 bp upstream of the ATG) for Cu-responsive genes. This result suggests the possibility of designing strategies to test Cu-deficiency by fusing *pFSD1* to amenable reporters in agricultural practices.

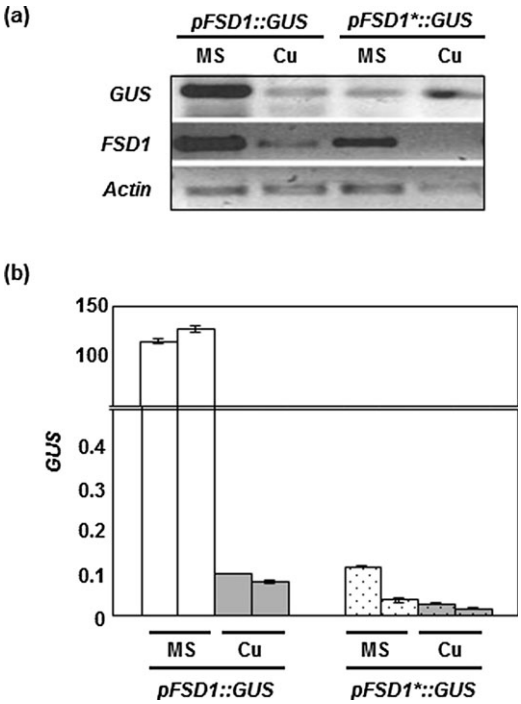

Fig. 4 Functionality of the *FSD1* putative Cu deficiency-responsive *cis* regulatory element in *Arabidopsis* seedlings. Expressions of the *GUS* and *FSD1* genes. The total RNA from the 6-day-old *pFSD1::GUS* (plain bars) and *pFSD1*::GUS* (−4GTAC boxes in ≤65 bp included in the 500 bp upstream of the gene transcriptional start; dotted bars) transgenic seedlings grown on commercial MS medium without (MS; white bars) and with Cu 10 μM (Cu; grey bars) was isolated and retrotranscribed to cDNA. The sqPCR products for *GUS* and *FSD1* are shown in (a) with *Actin* mRNA as the loading control. In (b), the mRNA amounts of *GUS* were measured by qPCR with specific primers and were normalised to the *UBQ10* expression for two independent transgenic lines. mRNA levels are expressed as percentages in relation to a *pFSD1::GUS* seedling under low Cu conditions. Values are the means ± SD of at least three biological replicates.

Defining nutritional Cu ranges at the molecular level

In order to further characterise the concentration range within which the transcriptional regulation of some of these genes takes place, seedlings were grown in different media with Cu concentrations ranging from 0 to 0.7 μM. The expression of two well-known Cu deficiency-responsive genes, *FSD1* and *CCH*, was checked by qPCR (Fig. 5a). Moreover, two genes whose expression is already known to be induced by high Cu, *CSD1* and *CCS* were also analysed within the same concentration range (Fig. 5b). *FSD1* exhibited 50% of its maximum expression at around 0.2 μM Cu (Fig. 5a). The 50% maximum expression of cuprochaperone *CCH* was around 0.3 μM Cu (Fig. 5a). *CSD1* and *CCS* exhibited their 50% maximum expression at around 0.25 and 0.3 μM Cu, respectively (Fig. 5b). The expression patterns of these genes clearly delineated the transcriptional changes occurring within the Cu deficiency range and further suggest Cu concentrations of around 0.5 μM to be the limit between sufficiency and deficiency, where plants sense low Cu supply and initiate molecular responses to alleviate it.

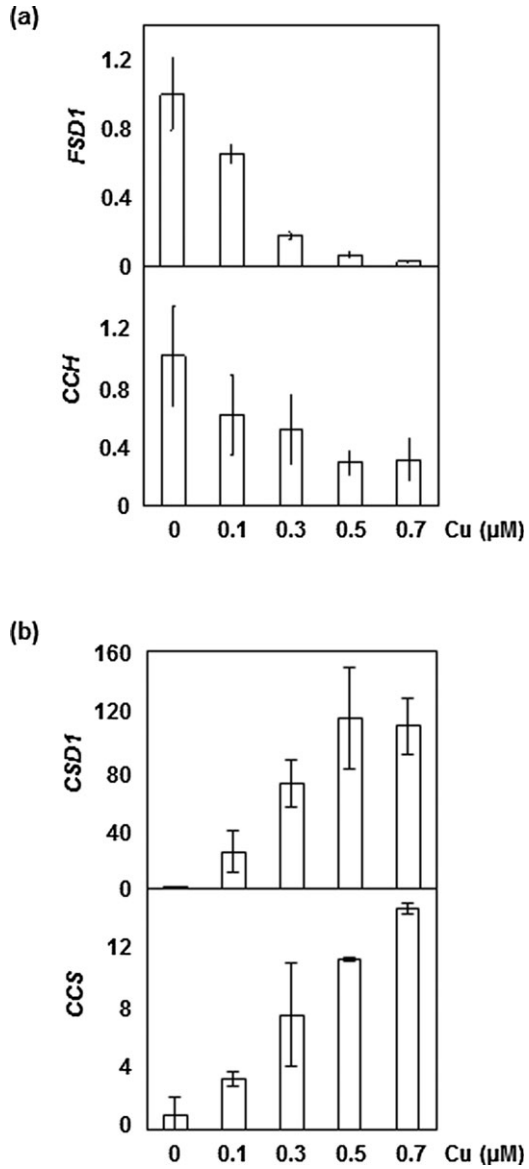

Fig. 5 Expression of the Cu status markers in the *Arabidopsis* seedlings grown on different Cu-supplemented media. Relative expressions of the Cu deficiency markers *FSD1* and *CCH* (a), and the Cu excess markers *CSD1* and *CCS* (b). The total RNA from the 6-day-old WT seedlings grown on homemade MS media with different Cu concentrations was isolated and retrotranscribed to cDNA. The mRNA amounts of each gene were measured by qPCR with specific primers and were normalised to the *UBQ10* expression. mRNA levels are expressed in relative units to the 0 μM Cu-supplemented medium. Values are the means ± SD of at least three biological replicates.

In light of this, the unknown and most Cu-regulated expressions of the overrepresented genes analysed under Cu deficiency (*CAS*) and Cu excess (*SDH1-2*) conditions (Fig. 2 and 3) were tested within a wide Cu concentration range (Fig. 6a and b). As shown in Fig. 6a, the *CAS* expression increased under low Cu *vs.* high Cu conditions, with a similar pattern to the previous well-established Cu deficiency homeostasis markers (Fig. 5a). Furthermore, the CAS expression under different metal status indicated the Cu specificity of this response since other metals in excess had no inhibitory effects on induction with the Cu-deficient MS medium (Fig. 6c). Conversely, excess Fe increased its expression, while it lowered under Fe deficiency conditions (Fig. S6, ESI†). Interestingly, the *CAS* expression responded to both metals similarly to *FSD1*,[22,50] further reinforcing the proposed Cu–Fe interaction.[8,11,50] CAS is required for photoacclimation in *Chlamydomonas reinhardtii*, and particularly for proper stomatal regulation in response to raised external Ca^{2+}.[51,52] These results suggest chloroplast to be a sensor of unbalanced Cu supply where signalling molecules, such as Ca^{2+}, ROS and ABA, may participate in global plant responses to Cu status.

A possible cross-talk involving the antioxidant system, external Ca^{2+} and abscisic acid (ABA) hormone signalling has been proposed.[53] Indeed, several genes related to ABA signalling were overrepresented under high Cu conditions (Table S1, ESI†). Moreover Ca^{2+} fluxes and ROS production have already been reported to participate in both Cu signalling[54] and circadian rhythm regulation.[55] In light of this, some Cu-regulated categories in our microarray well fit processes that have been previously reported to be circadian clock-regulated: ion homeostasis, osmotic stress responses and cell growth.[56,57]

The *SDH1-2* expression increased at Cu levels of 5 μM in the growth medium (Fig. 6b), which represented Cu supra-optimal supply (>20 μg Cu per gram of dry weight) (Fig. 1).[31] Moreover, this induction was highly specific to Cu (Fig. 6d). A plausible explanation for the SDH1-2 function may be envisaged from the fact that Cu^{2+} has been reported to inhibit all the succinate-linked partial electron transfer reactions through its binding to active thiol groups before the site of entry of electrons from ascorbate.[58] Thus, as previously suggested, *SDH1-2* could contribute to the synthesis of complex II in order to restore mitochondrial electron transport.[59] Mitochondria Cu homeostasis has been proven a key player in senescence and life span in other organisms, such as *Caernorhabditis elegans.*[60] In agreement, our results indicate that mitochondria may be involved in the complex network of responses of higher plants to Cu status. Furthermore, these data indicate *SDH1-2* as a new specific gene marker for mild Cu stress, which may prove useful for the detection of overdoses in Cu-containing agronomical treatments.

Experimental

Plant growth conditions and treatments

A. thaliana plants, ecotype Col 0, and *spl7* knockout plants[10] were grown as previously described.[33] The $MS_{1/2}$ medium (MS)[30] was either commercial (Sigma; www.sigmaaldrich.com) or prepared in the laboratory as follows: macronutrients 12.5 ml (NH_4NO_3 825 mg l^{-1}, KNO_3 950 mg l^{-1}, $MgSO_4{\cdot}7H_2O$ 90.35 mg l^{-1}, KH_2PO_4 85 mg l^{-1} and $CaCl_2$ 166.25 mg l^{-1}), micronutrients 0.5 ml (H_3BO_3 3.1 mg l^{-1}, $MnSO_4{\cdot}H_2O$ 8.45 mg l^{-1}, $ZnSO_4{\cdot}7H_2O$ 4.3 mg l^{-1}, $NaMoO_4{\cdot}2H_2O$ 0.125 mg l^{-1} and $CoCl_2{\cdot}6H_2O$ 0.0125 mg l^{-1}), Fe–EDTA 2.5 ml ($FeSO_4{\cdot}7H_2O$ 13.9 mg l^{-1} and $Na_2EDTA{\cdot}2H_2O$ 18.63 mg l^{-1}), KI 1.1 ml (0.41 mg l^{-1}), MES 0.5 g l^{-1}, sucrose 10 g l^{-1} and agar 8 g l^{-1}; pH 5.7 with KOH. Variable $CuSO_4$ concentrations were added

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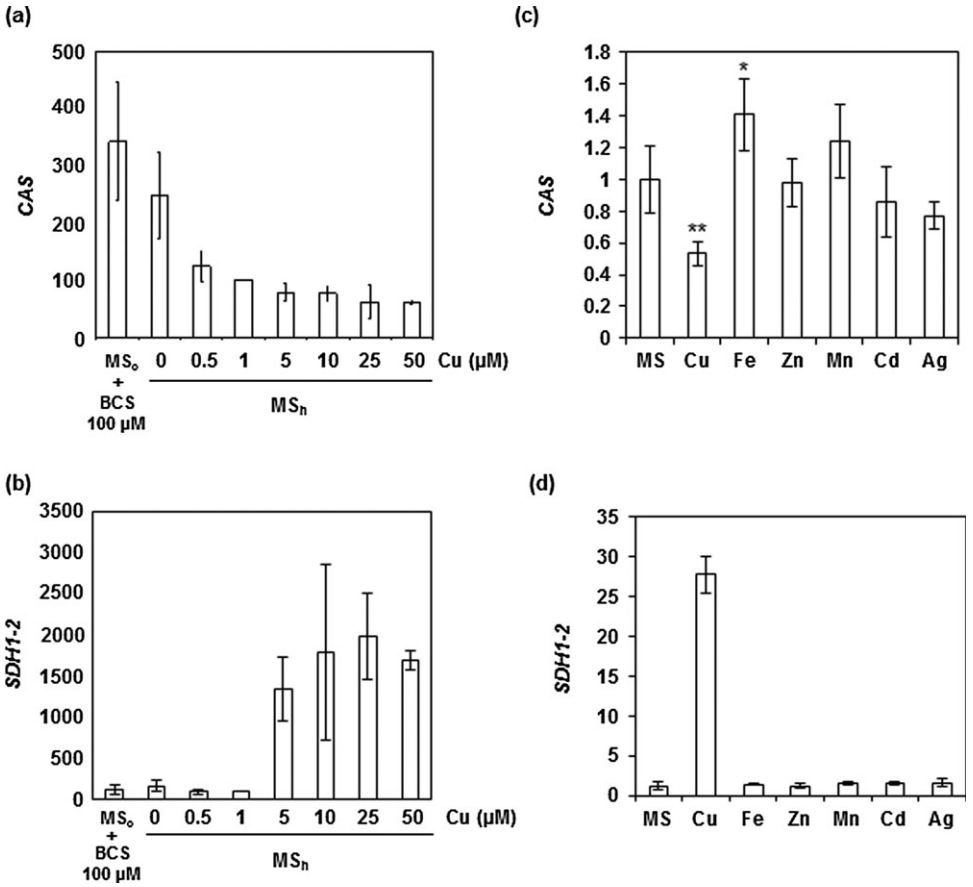

Fig. 6 Expression of the *CAS* and *SDH1-2* genes in the *Arabidopsis* seedlings grown on different metal-supplemented media. Relative expressions of the *CAS* (a and c) and *SDH1-2* (b and d) genes. The total RNA from the 6-day-old WT seedlings grown on MS media (commercial or homemade) supplemented with BCS or Cu, as indicated (a and b) or homemade MS media with 30 µM of different metals (c and d), was isolated and retrotranscribed to cDNA. The mRNA amounts of each gene were measured by qPCR with specific primers and were normalised to the *UBQ10* expression. mRNA levels are expressed as percentages in relation to the 1 µM Cu-supplemented medium (a and b) or as relative units to the MS (c and d). Values are the means ± SD of at least three biological replicates. Asterisks represent significant differences in relation to homemade MS medium (*$P < 0.05$; **$P < 0.01$).

when indicated. Media were supplemented with BCS 100 µM or ferrozine 300 µM for the Cu- or Fe-deprived media, respectively. Plants were grown under a long day photoperiod (16 h light-23 °C/8 h dark-16 °C; 16L/8D), except for the gene expression analysis by real-time quantitative PCR on different Cu-supplemented media where plants were grown under neutral photoperiod (12 h light-23 °C/12 h dark-16 °C; 16L/8D). Samples were collected at around 4 h after the light has been switched on. Soil-grown plants were watered with MS solution and $CuSO_4$ concentrations, as indicated. Lipid peroxidation was measured as MDA content.[61] Chlorophyll content was determined by the trichlorometric method.[62]

Gene expression by semi-quantitative and real-time quantitative PCR

Total RNA was isolated from *A. thaliana* seedlings with Trizol Reagent (Ambion). RNA was quantified by UV spectrophotometry and its integrity was visually assessed on ethidium bromide-stained agarose gels. Total RNA (1.5 µg) was first converted into cDNA by reverse transcription using SuperScript II reverse transcriptase (Invitrogen) and anchored oligo$(dT)_{15}$ (Roche). Semi-quantitative PCRs (sqPCR) were performed with specific oligonucleotides (Table S4, ESI†). Real-time quantitative PCRs (qPCR) were carried out with *SYBR-Green qPCR Super-Mix-UDG with ROX* (Invitrogen) and specific primers (Table S5, ESI†) in a *StepOnePlus Real-Time PCR System* (Applied Biosystems) under 1 cycle of 95 °C for 2 min and 40 cycles consisting in 95 °C for 30 s and 60 °C for 30 s. The results correspond to the comparative Ct (cycle threshold) method.

Metal accumulation measurements

Lyophilised samples were digested with HNO_3 and H_2O at 100 °C. Digested samples were diluted with H_2O milliQ (PURELAB Ultra; www.elgalabwater.com). Cu, Fe and Zn contents were analysed by inductively coupled plasma mass spectroscopy ICP-MS (Perkin Elmer Optima 3200RL; www.PerkinElmer.com). These measurements were taken at the *Unitat d'Anàlisi Elemental, Serveis Cientificotècnics* at the *Universitat de Barcelona*.

Obtaining transgenic chimeric plants

An *FSD1* promoter fragment (1193 bp) (*pFSD1*) and a modified promoter (1128 bp) with a 65-bp fragment deletion containing

four GTAC boxes (*pFSD1**) were fused with the coding region of the β-glucuronidase (GUS) reporter as follows. The *pFSD1* fragment was obtained from *A. thaliana* genomic DNA by PCR using the following specific primers, which introduce adequate restriction sites for cloning: FSD1 *HindIII* F/FSD1 *BamHI* R. Two fragments of 806 bp and 361 bp were obtained from *A. thaliana* genomic DNA by PCR by respectively using the following two pairs of specific primers: FSD1 *HindIII* F/FSD1-4GTAC R and FSD1-4GTAC F/FSD1 *BamHI* R, where FSD1-4GTAC F and R were designed to be complementary. The *pFSD1** fragment was obtained by using the two previous primers in a PCR due to the complementary region of both fragments. The obtained promoter fragments were cloned at the multicloning site of the *pFP101* vector[63] with HindIII and BamHI restriction enzymes. The C58 strain of *Agrobacterium tumefaciens*, transformed with this construct, was used to transform *A. thaliana* plants by following the floral dip protocol.[49] Transgenic plants were selected by seed fluorescence.

Gene expression analysis by long oligonucleotide microarrays

Long oligonucleotide microarrays were provided by Dr David Galbraith (University of Arizona, www.ag.arizona.edu/microarray/). The analysis was performed as described.[64] Three biological replicates (6-day-old seedlings grown under a 16L/8D photoperiod) were obtained for each treatment (commercial $MS_{1/2}$ with and without Cu 10 μM), and samples were taken consecutively at the same time during the photoperiod (4 h after light has been switched on) to avoid differences in the diurnal or circadian regulations of the gene expression. For each biological replicate, two microarrays were hybridised for six microarrays. Only the spots for which valid data were obtained for at least four of the six hybridisations were selected. The data from two of the six microarrays were eliminated because of detected hybridisation problems. The median $\log_2$ ratio (by comparing high Cu status to low Cu status) was calculated. The gene list, ordered according to the mean $\log_2$ ratio, was analysed using the FatiScan (GEPAS, Gene Expression Pattern Analysis Suite, v3.1; http://babelomics.bioinfo.cipf.es/fatiscan/cgi-bin/fatiscan.cgi) with 30 partitions and a two-tailed Fisher's exact test with an adjusted *p*-value after correcting for multiple testing following the FDR procedure[65] and the GeneCodis2.0 (http://genecodis.dacya.ucm.es/) programmes. Microarray raw data were deposited in the NCBI GEO database under accession number GSE13114.

Computer-assisted sequence analysis

The theoretical promoter sequence analysis was performed by the PLACE Web Signal Scan (www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) and Patmatch from TAIR (www.arabidopsis.org).

Statistical analysis

Statistical analysis of the relative expressions was performed by applying the comparison of the relative gene expressions ($P < 0.05$), and was carried out using two-way ANOVA for the remaining parameters, while means were compared by the Duncan test ($P \leq 0.05$) with the *InfoStat software*, *2010 version* (www.infostat.com.ar), or by the *t*-test with the *GraphPad software* (www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD).

The expression levels for the microarrays were statistically analysed in a one-class response using a significance analysis of microarray (SAM[66]), a criterion for the selection of fold change $\log_2 \geq |1|$ and a false discovery rate (FDR) of $<2\%$.

Conclusions

The current study shows a microarray analysis of *A. thaliana* plants grown under mild Cu deficiency and excess conditions which underscore gene expression changes under nutrient sufficiency limits. A CuAt database was created to compile relevant data on Cu homeostasis in plants and responses to different Cu status. This study uncovers a new mild Cu deficiency responsive gene, chloroplast Ca^{2+} transducer *CAS*. Reiterative GTAC boxes in proximal promoter regions may act as a *cis* element responsible for gene expression induction under Cu-deficiency conditions, as shown herein for *FSD1* regulation. In the case of mild Cu excess, the flavoprotein subunit of succinate dehydrogenase in mitochondria *SDH1-2* is proposed as a new molecular marker that might facilitate prompt detection of Cu overdoses in crops treated with Cu-based fungicides or pesticides.

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