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Cd-induced Cu deficiency responses in *Arabidopsis thaliana*: are phytochelatins involved?

Running title: Cd-induced Cu deficiency responses

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ABSTRACT

Cadmium (Cd) exposure can disturb the homeostasis of essential elements. In *Arabidopsis thaliana*, Cd induces a squamosa promoter binding protein-like 7 (SPL7)-dependent Cu deficiency response. We investigated how Cd induces a Cu deficiency response. The Cu deficiency response consists of the active SPL7 transcription factor binding to GTAC motifs in promoters of among others several Cu transporters, a Cu chaperone, and cupro-miRNAs to regulate Cu homeostasis. We demonstrated that the addition of supplemental Cu to Cd-exposed *A. thaliana* plants diminished the Cu deficiency response in roots, while it even disappeared in leaves. Exposure of plants to Cd in combination with extra Cu reduced Cd levels in both roots and leaves resulting in an improved cellular oxidative state. Furthermore, we demonstrated a role for phytochelatins (PCs) in the Cd-induced Cu deficiency response, since it was reduced in roots of *cad1-3* mutant plants exposed to Cd. In conclusion, a working mechanism is provided in which it is suggested that Cd increases PC levels that can complex both Cd and Cu. This results in cellular Cu deficiency and subsequently the activation of SPL7 and hence the induction of the Cu deficiency response.

KEYWORDS

Cadmium, Copper, phytochelatins, SPL7, Arabidopsis, Cu deficiency, Cu homeostasis, metal stress

INTRODUCTION

Due to industrial and agricultural activities, toxic metals such as cadmium (Cd) are dispersed in many regions all over the world. Contamination of soils with Cd is a major adverse stress factor for the environment and a potential threat for all organisms. Furthermore, the uptake of Cd by plants, as primary producers, brings these toxic metals into the food chain, threatening human health.

Since Cd is non-essential, plants do not possess specific transporters for this metal. Instead, Cd enters root cells through transporters of essential nutrients like the nonspecific transporters ZIP/IRT1 (zinc-regulated transporter/iron-regulated transporter) and calcium channels (Clemens, 2006; Verbruggen et al., 2009). Once inside the cell, the distribution of Cd to cell organelles and throughout the plant is possible via several transporters like heavy metal ATPases (HMAs). In *Arabidopsis thaliana*, it was demonstrated that HMA3 is located in the tonoplast transporting Cd into the vacuole (Morel et al., 2009), while HMA2 and HMA4 can load Cd into the xylem for long-distance transport (Wong & Cobbett, 2009).

Since Cd will be transported throughout the plant, it can interfere with several cellular processes such as photosynthesis and respiration (DalCorso et al., 2008). Furthermore, Cd indirectly causes the generation of reactive oxygen species (ROS) resulting in an oxidative challenge (Cuypers et al., 2012; Sharma & Dietz, 2009). The presence of an antioxidative defence network, consisting of metabolites [*e.g.* GSH and ascorbate (AsA)] and enzymes [*e.g.* superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)], provides plants with the ability to cope with this Cd-induced oxidative stress. Furthermore, Cd can disturb the homeostasis of essential nutrients through competition with their uptake and with their binding sites in metalloproteins (DalCorso et al., 2008; Sharma & Dietz, 2009). For example, it was demonstrated that Cd disturbed iron (Fe) levels in shoots and roots and this was molecularly supported by increased transcript levels of *IRT1* and *FRO2* (ferric reductase oxidase) (Xu et al., 2015; Yoshihara et al., 2006). It is also proven that Cd interferes with Cu homeostasis, inducing a Cu deficiency response dependent on SPL7 (SQUAMOSA promoter binding protein like7), the master regulator transcription factor (TF) of Cu homeostasis (Gayomba et al., 2013; Gielen et al., 2016; Yamasaki et al., 2009). In this Cu deficiency response on the one hand, several Cu transporters, like COPT2 and ZIP2, are upregulated to increase Cu uptake. On the other hand, the so-called cupro-

miRNAs targeting transcripts of Cu-containing proteins are increased to reallocate Cu to essential proteins. Gielen *et al.* (2016) suggested that Cd possibly provokes Cu deficiency, thereby activating SPL7 and subsequently inducing the Cu deficiency response.

Therefore, it is important that plants possess defence strategies to limit free Cd. Once inside the cell, Cd is detoxified and sequestered to the least sensitive parts in the cell (*e.g.* vacuole) by binding to chelators like glutathione (GSH) and phytochelatins (PCs). Glutathione (γ-glu-cys-gly) has a thiol group, a favoured ligand of Cd, and polymerization of 2-11 moieties from GSH molecules by PC synthase (PCS) results in the formation of PCs. The need for this chelating capacity is high in Cd-exposed plants and therefore these plants increase their GSH and PC levels as demonstrated in several studies (Jozefczak et al., 2014; Sofo et al., 2013). Furthermore, *cad2* mutants with reduced GSH and PC levels and *cad1* mutants lacking PCs are hypersensitive to Cd (Cobbett, 2000; Howden et al., 1995; Jozefczak et al., 2015; Sofo et al., 2013). Therefore, this chelating capacity is very important for Cd tolerance and detoxification.

The aim of this study is to explore how Cd provokes Cu deficiency in *A. thaliana* and subsequently induces the Cu deficiency response. Therefore, we exposed wildtype plants to a Cd containing nutrient solution supplemented with extra Cu and monitored the impact of this exposure on metal concentration and transcript levels of SPL7-regulated genes and heavy metal transporters. Furthermore, we investigated the involvement of PC production in the Cd-induced Cu deficiency response by using a *cad1-3* mutant that is deficient in PC-synthase activity and thus the formation of PCs.

MATERIALS AND METHODS

Plant culture, exposure and harvest

The homozygosity of the *cad1-3* knockout mutant *A. thaliana* line (Col-0 background) was confirmed by PCR. Wild-type (WT; Col-0 background) and mutant seeds were surface sterilized and grown in hydroponic culture (Smeets et al., 2008) in the same conditions as described by Keunen *et al.* (2013*a*). After 19 days, the Hoagland solution was supplemented with 5 µM $CdSO_4$ alone or combined with extra $CuSO_4$ (0.5, 1 or 2 µM $CuSO_4$) and plants were harvested after an exposure time of 24 and/or 72

h. Copper deficient plants were grown from germination on in an adapted Cu deficient Hoagland solution (0.5 nM Cu). Root and leaf (entire rosette) samples were taken, snap frozen in liquid nitrogen and stored at -70°C prior to gene expression analysis. Samples for element analysis were dried prior to extraction.

Gene expression analysis

Frozen tissues (50-75 mg FW) were disrupted using two stainless steel beads and the Retsch Mixer Mill MM400 (Retsch, Belgium) under frozen conditions. Total RNA was extracted using the Mirvana Kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions (Total RNA isolation). cDNA samples have an RNA input of 1 µg that was DNase treated (Turbo DNA-freeTM kit, Life Technologies) and reverse transcribed using the PrimeScript RT reagent Kit (Perfect Real Time, Takara Bio inc., Westburg, The Netherlands) according to manufacturer's instructions. A 10-fold dilution of cDNA was made using 1/10 diluted TE buffer (1 mM Tris-HCL, 0.1 mM EDTA, pH 8.0, Sigma-Aldrich, Belgium) and stored at -20°C.

Quantitative PCR analysis was performed with the 7500HT Fast Real-Time PCR System (Life Technologies). Primers were designed using primer express 2.0 and a BLAST was performed (http://www.arabidopsis.org/Blast/index.jsp) to check specificity (Table S1). A two-fold dilution series of a pooled sample (all cDNAs of the experiment) was used to create a standard curve for the evaluation of primer efficiencies that were accepted when they were within a range between 80% and 100% (measured over 6 dilution points). PCR amplifications were performed at universal cycling conditions and contained Fast SYBR Green Master Mix, 300 nM of a gene-specific forward and reverse primer, and 2 µl of the diluted cDNA in a final volume of 10 µl.

Specifically for the gene expression analysis of mature miRNAs, a multiplex reverse transcription and Real-time qPCR were performed using Taqman microRNA assays (Life Technologies).

All relative expression levels were calculated as $2^{-\Delta Cq}$ and normalized by the geometric average of $2^{-\Delta Cq}$ values of minimum three reference genes selected by the GrayNorm algorithm (Remans et al., 2014). Data of treatment effects are expressed relative to the control of its own genotype set at 1.00. For some genes expression was not detected after 40 cycles under control conditions. In that case,

cyclic threshold was set at 40 for the control samples and thereby treatment effects could be calculated and expressed relative to the control. The calculated and represented treatment effects are therefore minimal fold changes when expression was undetermined after 40 cycles under control conditions. All details of the workflow according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines as described by Bustin *et al.* (2009) are shown in Table S2.

Element analysis

Fresh root samples (175-400 mg FW) were washed for 15 min with 10 mM $Pb(NO_3)_2$ at 4°C and rinsed with distilled water, while leaf samples (400-1000 mg FW) were only rinsed with distilled water. Samples were oven-dried (60°C for 3 weeks), weighed, digested with 70% HNO_3 in a heat block and dissolved in 5 ml of 2% HCl. Copper and Cd concentrations were measured via inductively coupled plasma-atomic emission spectrometry (ICP-OES, Agilent Technologies, 700 series, Belgium). Blanks (only HNO_3) and standard references (NIST Spinach 1570a) were included.

Statistical analysis

Statistical analysis was performed using RStudio (version 0.99.485; R Foundation for Statistical Computing, Vienna, Austria). Normal distribution of the datasets was tested using the Shapiro-Wilk test and homoscedasticity was evaluated with the Bartlett's test. If necessary, transformations of the datasets were applied. Gene expression data were always log transformed. Significant differences were determined using ANOVA test and Tukey correction. If the assumption of normality was not fulfilled, a non-parametrical ANOVA test (Kruskal-Wallis) and correction with pairwise Wilcoxon rank sum test was applied.

RESULTS

Effects of the combined application of Cd and supplemental Cu to elevate the Cu deficiency response

Previously it was demonstrated that Cd induces a Cu deficiency response (Gielen et al., 2016). To further explore and get more insight in the Cd-induced Cu deficiency responses, plants were grown

under Cu deficiency or were exposed to 5 µM Cd alone or supplemented with extra Cu (0.5, 1 or 2 µM) for 72 h.

Plant fresh weight decreases with increasing Cu concentration

The fresh weight (FW) of leaves and roots was determined in plants grown under control conditions and after 72 h exposure to 5 µM Cd alone or supplemented with extra Cu and in plants grown under Cu deficiency from germination on (Figure 1). Copper deficient plants showed no differences in leaf and root FW in comparison to control plants. Exposure to 5 µM Cd significantly decreased root FW, yet not leaf FW. However, additional Cu in combination with 5 µM Cd decreased leaf and root FW with increasing Cu, although no differences between the addition of 1 and 2 µM Cu extra were observed.

Additional Cu provokes alterations in Cu and Cd concentration

Plants grown under Cu deficiency had significantly lower Cu levels in both roots and leaves (Table 1). Significantly lower Cu levels were also found in the leaves after exposure to Cd as well as after exposure to Cd supplemented with 0.5 µM Cu. However, adding higher amounts of Cu (1 and 2 µM Cu) together with Cd did not lead to leaf Cu concentrations different from the control plants (Table 1). On the contrary, in the roots of Cd-exposed plants, Cu concentration was significantly increased and adding extra Cu dose-dependently increased Cu levels even more. In both leaves and roots, the Cd concentration was significantly diminished in plants exposed to Cd supplemented with extra Cu in comparison to plants exposed to Cd alone (Table 1).

The translocation of Cd from roots to shoots was significantly reduced in plants exposed to 5 µM Cd supplemented with 1 or 2 µM Cu in comparison to plants exposed to Cd only (Figure 2).

Gene expression levels of SPL7-dependent genes

Since the transcription factor SPL7 is involved in the response to Cd exposure and in Cu homeostasis (Gayomba et al., 2013; Gielen et al., 2016), transcript levels of SPL7-dependent genes were

171 determined in leaves and roots. The expression level of *FSD1* (Fe superoxide dismutase) was
172 enhanced in both leaves and roots of plants grown under Cu deficiency or after exposure to 5 µM Cd
173 (Figure 3). However, this response disappeared in the leaves in the combined application of Cd and
174 supplemental Cu. Similarly in the roots, although transcript levels of *FSD1* were still increased in
175 comparison to control when extra Cu was supplied, its expression was significantly diminished in
176 comparison to plants exposed to Cd alone (Figure 3). In general, similar changes in gene expression
177 levels were found for the SPL7-regulated miRNAs (miR397a, miR398b/c and miR857) and SPL7-
178 regulated genes [*CCH (Cu chaperone)*, *COPT2* and *ZIP2*] (Figure 3).
179
180 ***Changes in transcription levels of heavy metal transporters***
181 To determine the involvement of heavy metal transporters in the changed Cu and Cd concentration
182 after exposure to Cd supplemented with extra Cu, transcript levels of several HMAs were analysed. In
183 the leaves, there were no changes in the transcript levels of these HMAs, except for a significantly
184 enhanced expression of *HMA5* after exposure to 5 µM Cd alone or in combination with 0.5 µM Cu
185 (Table 2). In the roots, the expression levels of *HMA4*, *HMA5* and *HMA7* were significantly higher
186 after exposure to 5 µM Cd and this increase diminished (*HMA5*) or even disappeared (*HMA4* and
187 *HMA7*) in plants exposed to 5 µM Cd combined with 0.5 µM Cu. However, the addition of higher Cu
188 concentrations (1 and 2 µM Cu) in combination with 5 µM Cd again led to increased transcript levels
189 of *HMA5* and *HMA7*, but reduced the expression levels of *HMA2* and *HMA3* (Table2).
190
191 ***Oxidative stress hallmark genes***
192 To know the general oxidative stress state in plants exposed to 5 µM Cd combined with supplemental
193 Cu, transcript levels of hallmark genes for oxidative stress were determined (Gadjev et al., 2006). In
194 the leaves, the expression levels of four out of five hallmark genes were upregulated after exposure to
195 5 µM Cd (Table 3). However, this induction diminished or even disappeared when plants were
196 exposed to Cd in combination with supplemental Cu (Table 3). In the roots, exposure to 5 µM Cd
197 increased the transcript levels of all five oxidative stress hallmark genes (Table 3). This increased
198 expression was maintained in plants exposed to 5 µM Cd combined with supplemental Cu for *UPOX*

(UPregulated by OXidative stress) and the unknown *AT1G19020* gene. In contrast, the Cd-induced transcript levels diminished for the genes *Defensin-like* and *TIR-class*, and disappeared for the unknown *AT1G05340* gene when plants were exposed to 5 µM Cd in combination with 0.5 µM Cu (Table 3). However, the addition of higher Cu concentrations (1 and 2 µM) in combination with 5 µM Cd significantly increased these transcript levels in comparison to controls and were similar to the Cd-induced transcript levels (Table 3).

The involvement of phytochelatin production in the Cd-induced Cu deficiency response

To determine the involvement of PC production in the Cd-induced Cu deficiency responses, responses were compared in WT versus *cad1-3* mutant plants after exposure to 5 µM Cd for 24 h and 72 h.

Cd-induced effects on growth

Under control conditions, no differences in leaf and root fresh weight (FW) between wildtype and *cad1-3* mutant plants were observed (Figure 4). For both genotypes, there were no significant effects on the FW of leaves and roots when plants were exposed to 5 µM Cd for 24 h. However, while exposure to 5 µM Cd for 72 h did not significantly change FW of leaves and roots in wildtype plants, FW was significantly reduced in both leaves and roots of *cad1-3* mutant plants (Figure 4).

Cd-induced effects on elemental concentration

In wildtype plants, the Cu concentration in the roots was significantly increased after Cd exposure for 72 h, while in the *cad1-3* mutant plants Cu concentration was already significantly increased after 24 h Cd exposure (Table 4). Furthermore, the Cu concentration in the roots was significantly higher in Cd-exposed *cad1-3* mutant plants in comparison to wildtype plants. Similarly, Cd-exposed *cad1-3* mutant plants had a higher Cd concentration in the roots then wildtype plants (Table 4). Potassium (K) concentration was significantly decreased in the roots of the Cd-exposed *cad1-3* mutant plants in comparison to *cad1-3* control plants, while K concentration didn't change in wildtype plants (Table 4). Furthermore, the Cd concentration in the shoots (data not shown) and the Cd translocation from roots

to shoots was significantly lower in *cad1-3* mutant plants in comparison to wildtype plants when plants were exposed to 5 µM Cd for 24 h and 72 h (Figure 5).

Opposite Cd-induced gene expressions in the cad1-3 mutant in comparison to wildtype plants

Phytochelatins are already important early on in roots upon Cd exposure (Jozefczak et al., 2014). To unravel a possible link between PCs and the Cd-induced Cu deficiency response, we focused on alteration of gene expression in roots. Since *cad1-3* mutant plants severely suffered from exposure to Cd for 72 h (Figure 4 and Table 4), gene expression analysis was performed on plants exposed to Cd for 24 h. In wildtype plants, the transcript levels of SPL7-regulated genes were significantly upregulated after exposure to 5 µM Cd (Table 5) similar to what is seen in Figure 1. In contrast to the wildtype, exposure to 5 µM Cd in the *cad1-3* mutant did not alter these expression levels or even led to a downregulation of these transcripts (Table 5).

Changes in gene expression of Cd transporters

The expressions of both *HMA2* and *HMA4* were significantly lower in Cd-exposed *cad1-3* mutant plants in comparison to their control, while *the* expression was not significantly altered in wildtype plants (Figure 6A and B). Transcript levels of *HMA5* were significantly increased in Cd-exposed WT plants in comparison to WT control plants, while no differences were demonstrated for *cad1-3* mutant plants (Figure 6C).

DISCUSSION

A major mechanism by which Cd exerts its toxicity is the disturbance of the homeostasis of essential elements. Previous results have indicated a Cd-induced disturbance of the Cu homeostasis in *A. thaliana* (Gayomba et al., 2013; Gielen et al., 2016). Cadmium exposure changed the Cu concentration in leaves and roots and this was accompanied by a Cu deficiency response at transcriptional level. It was demonstrated that this Cd-induced Cu deficiency response is dependent on the transcription factor SPL7 (Gayomba et al., 2013; Gielen et al., 2016), the master regulator of Cu homeostasis (Yamasaki et al., 2009). In Cu limited conditions, SPL7 activates this response, which includes on the one hand

the enhanced Cu uptake by increasing the Cu transporters *COPT2* and *ZIP2*, and on the other hand the increased expression of cupro-miRNAs to reduce their Cu-containing targets in order to reallocate Cu to essential proteins (Yamasaki et al., 2009). Gayomba *et al.* (2013) hypothesized that this Cu deficiency response is required for basal Cd tolerance, since *spl7* mutant plants were hypersensitive to Cd. Furthermore, Gielen *et al.* (2016) illustrated that the addition of extra Cu to Cd-exposed *A. thaliana* plants led to the disappearance of the Cu deficiency response in leaves. Therefore, they suggested that Cd provokes Cu deficiency thereby activating SPL7 resulting in the induction of the Cu deficiency response. However, how Cd provokes Cu deficiency and subsequently activates SPL7 is yet to be unravelled.

In this study, we demonstrate that the disappearance of the Cd-induced Cu deficiency response when supplemental Cu is supplied to Cd-exposed plants, does not only occur in leaves. Also in the roots the transcript levels of the SPL7-regulated genes are decreased when extra Cu is provided in comparison to exposure to Cd alone (Figure 3). The leaves of plants exposed to Cd showed a decreased Cu concentration (Table 1) that could result in the activation of SPL7 and induction of this Cu deficiency response. The supply of extra Cu in combination with Cd exposure restored Cu levels to control conditions (Table 1), abolishing the Cu deficiency, which consecutively resulted in the disappearance of the SPL7-regulated Cu deficiency response. However, plants exposed to 5 µM Cd together with 0.5 µM Cu still showed a reduced Cu concentration in the leaves, though the Cu deficiency response was not present anymore (Figure 3). In addition, although the Cu concentration in the roots increased after exposure to Cd alone and even more when extra Cu was supplied (Table 1), the Cu deficiency response was still observed in the roots. Nevertheless, the increased transcript levels of SPL7-regulated genes after Cd exposure diminished when extra Cu was supplied (Figure 3). It should be noted however that the Cd concentration in both leaves and roots significantly reduced when additional Cu was supplemented to Cd-exposed plants. Probably, this extra Cu competes with Cd uptake resulting in lower Cd levels in the roots and diminished Cd transport to the shoots (Table 1 and Figure 2).

The disturbance in Cu homeostasis and altered Cd levels in plants exposed to a combination of Cd and Cu raises the question if heavy metal transporters are involved in these changes, since they play a role

in the detoxification of non-essential heavy metals. HMA4 is located in the plasma membrane loading Cd into the xylem (Verret et al., 2004). Transgenic plants overexpressing HMA4 had an increased Cd tolerance and elevated Cd levels in the shoots, while an *hma4* mutant showed a high Cd sensitivity (Verret et al., 2004). Wong & Cobbett (2009) demonstrated that the *hma4* mutant as well as the *hma2/hma4* double mutant accumulated respectively only 40% and 3% Cd in shoots in comparison to wildtype plants. The HMA2 and HMA4 transporters are therefore the major mechanisms to translocate Cd to the shoots (Wong & Cobbett, 2009). Our results demonstrated that Cd exposure increased the expression of *HMA4* in the roots, while supplying extra Cu reduced the expression of *HMA4* back to control levels (Table 2). Possibly, this caused a reduction in the Cd translocation to the shoots when supplemental Cu is supplied to Cd-exposed plants (Table 1). The expression of *HMA5*, another plasma membrane localized transporter, was also increased in Cd-exposed plants (Table 2). HMA5 transports Cu to the apoplast and is probably involved in Cu xylem loading (Andrés-Colas et al., 2006). It was demonstrated that *HMA5* expression increased under Cu excess and that the Cu concentration in the roots of an *hma5* mutant was higher, suggesting a role for HMA5 in Cu detoxification in the roots (Andrés-Colas et al., 2006). In our study, Cd exposure increased Cu levels in the roots (Table 1), probably leading to the increased expression of *HMA5* (Table 2) to improve Cu detoxification in the roots and possibly to replenish the reduced Cu levels in the shoots. As Cu levels increased even more in roots when extra Cu was supplied to Cd-exposed plants, the increased *HMA5* expression in the roots remained present (Table 2). This means that different metal transporters respond in a metal specific way in roots of Cd-exposed plants. Nevertheless, the Cu deficiency response does not seem to solely rely on the cellular Cu concentration. Whereas sufficient Cu was present, Cd still induced a Cu deficiency response thereby stimulating Cu uptake resulting in excess Cu levels in the roots. This subsequently led to increased *HMA5* expression to reduce root cellular Cu levels. In conclusion, it is obvious that HMAs are involved in detoxifying Cu and Cd levels, but a direct connection to the Cd-induced Cu deficiency remains to be revealed, but is clearly not simply depending on the Cu concentration only.

It is clear that Cd-exposed plants, and certainly when extra Cu is supplied, invest a significant portion of their energy in the detoxification of and defence against these metals and their adverse effects.

Consequently growth is reduced in a dose-dependent manner when plants are exposed to Cd with extra Cu, which is demonstrated by the decreased fresh weights of leaves and roots (Figure 1). At the cellular level however, leaves of these exposed plants handle this combined metal stress quite well since oxidative stress levels diminished in comparison to single Cd-exposed plants (Table 3). On the contrary, roots of plants exposed to Cd in combination with extra Cu appeared to suffer more than in single Cd-exposed plants. In general, combined exposures significantly increased transcript levels of the oxidative stress hallmark genes in the roots in comparison to control plants (Table 3). However, a differentiation should be made between Cd-exposed plants supplemented with on the one hand 0.5 µM Cu and on the other hand supplemented with 1 or 2 µM Cu. The addition of 0.5 µM Cu to Cd-exposed plants decreased transcript levels of the oxidative stress hallmark genes in the roots in comparison with plants exposed to Cd alone (Table 3), which indicates that the cellular oxidation state had improved. In contrast, 1 or 2 µM Cu supplemented to Cd-exposed plants showed no difference in transcript levels of these oxidative stress hallmark genes in comparison to plant roots exposed to Cd alone, and even an increasing trend was seen for *Defensin-like* and *TIR-class* transcript levels (Table 3). Therefore, it can be concluded that adding a low amount of Cu to Cd-exposed plants improves the cellular oxidation state of the plants, while higher amounts of Cu evoked Cu toxicity in plant roots.

Although the Cu deficiency response disappeared in the leaves and diminished in the roots when extra Cu was supplied to Cd-exposed plants, Cd was still present in leaves and roots, though to a lesser extent. An important defence strategy of plants against Cd is the complexation and sequestration through PCs. The preferred ligands of Cd and Cu are thiols (SH-groups), which are multiple times present in PCs. It is demonstrated that Cd increases PC levels in *A. thaliana under similar conditions as our experiments* (Jozefczak et al., 2014) and that *cad1-3* mutants lacking PCs are hypersensitive to Cd (Howden et al., 1995; Sofo et al., 2013). Besides, exposure to excess Cu, from which it is known to be detoxified by metallothioneins (Cobbett & Goldsbrough, 2002; Guo et al., 2008), also resulted in increased PC levels in *Arabidopsis* seedlings (Sofo et al., 2013). From that, we hypothesized that the PCs induced by Cd can also complex Cu resulting in a Cu deficiency response, though Cu concentration is strictly not limited in the plant. To investigate this hypothesis, a *cad1-3* mutant was used which is defective in PC synthesis (G/C nucleotide exchange in the *PCS1* locus). Cadmium-

exposed wildtype and *cad1-3* mutant plants were compared for their transcript levels of the SPL7-dependent Cu deficiency response genes and for their metal concentration. Since roots are in direct contact with Cd when exposed and since Jozefczak *et al.* (2014) demonstrated in *A. thaliana* the presence of PCs already 2 h after exposure to Cd in roots, we focused on the roots to explore a possible role for PCs in the Cu deficiency response. The *cad1-3* mutant plants, lacking the so important PCs for Cd detoxification, were severely suffering when exposed to Cd for 72 h, which was reflected in the strongly decreased FW of leaves and roots (Figure 4) and K leakage in roots probably as a result of membrane damage (Table 4). Therefore, gene expression analysis was only performed on plants exposed during 24 h to Cd and discussed in relation to metal concentration and translocation. The Cu and Cd concentrations in the Cd-exposed roots of the *cad1-3* mutant were significantly elevated in comparison to the concentrations in Cd-exposed wildtype roots (Table 4). However, a lower metal uptake in *cad1-3* mutants was expected since *COPT2* and *ZIP2* expression levels significantly decreased in these plants in contrast to the strong increases in wildtype plants (Table 5). Translocation of Cd from root to shoot was also strongly diminished in the *cad1-3* mutant in comparison to wildtype plants (Figure 5). Since HMA2 and HMA4 are important transporters for Cd translocation (Wong & Cobbett, 2009), the expression levels of both transporters were also analysed. The transcript levels of both transporters were significantly decreased in the Cd-exposed *cad1-3* mutant plants (Figure 6A and B), which could explain the reduced Cd translocation in the *cad1-3* mutant plants (Figure 5). Transcript levels of *HMA5* were increased in Cd-exposed WT plants stimulating Cu translocation to the shoots and therefore decreasing Cu levels in roots, while the higher Cu levels in the roots of *cad1-3* mutant plants in comparison to WT plants upon Cd exposure (Table 4) are probably the result of the unaltered expression level of *HMA5* (Figure 6C).

While in the roots of wildtype plants Cd exposure significantly increased the expression levels of SPL7-dependent genes, the opposite occurred in the *cad1-3* mutant plants with significantly lower transcript levels of these genes (Table 5). Probably in the *cad1-3* mutant, free Cu levels are too high, deactivating SPL7 and subsequently downregulating the Cu deficiency response. This was also demonstrated by Gielen *et al.* (2016) when *A. thaliana* seedlings were exposed to excess Cu resulting in a downregulation of genes involved in the Cu deficiency response.

In conclusion, we propose a working mechanism illustrated in Figure 7 in which the Cd-induced increase of PCs also partly sequester Cu, provoking cellular Cu deficiency. This subsequently results in the activation of the transcription factor SPL7 and the induced transcription of its downstream targets resulting in a Cu deficiency response (Figure 7). The activation of the Cd-induced Cu deficiency response is therefore not caused by the real Cu concentration, but by the Cu sensing in plants. However, a major question about the mode of action of how (Cd-induced) Cu deficiency activates SPL7 remains to be explored.

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451

TABLES

Table 1. Copper and Cd concentrations in leaves and roots of A. thaliana. *Arabidopsis seedlings were either continuously grown under control conditions or Cu-deficiency or nineteen-days-old plants were exposed to 5 µM $CdSO_4$ alone or in combination with additional $CuSO_4$ (0.5, 1 or 2 µM Cu extra in comparison to control Hoagland solution) for 72 h. Copper and Cd* concentrations *are presented in mg per kg dry weight (mg kg^{-1} DW). Data are mean ± S.E. of 6 biological replicates. Significant differences ($P<0.05$) after one-way ANOVA test and Tukey correction (leaves) or after a non-parametrical ANOVA test (Kruskal-Wallis) and correction with pairwise Wilcoxon rank sum test (roots) are indicated with different letters.*

	Cu concentration		**Cd concentration**	
	Leaves	**Roots**	**Leaves**	**Roots**
Control	7.97 ± 0.46^{a}	11.34 ± 1.21^{a}	-	-
Cu deficiency	3.22 ± 0.45^{b}	1.54 ± 0.70^{b}	-	-
5 µM Cd	5.03 ± 0.17^{c}	36.01 ± 1.78^{c}	1067.29 ± 58.55^{a}	1713.73 ± 89.12^{a}
5 µM Cd + 0.5 µM Cu	4.73 ± 0.19^{bc}	307.81 ± 74.94^{d}	321.60 ± 56.06^{b}	952.81 ± 59.40^{b}
5 µM Cd + 1 µM Cu	7.51 ± 0.54^{a}	568.80 ± 55.53^{d}	128.25 ± 9.90^{c}	1055.81 ± 142.25^{b}
5 µM Cd + 2 µM Cu	9.13 ± 0.33^{a}	778.25 ± 56.18^{e}	169.80 ± 31.87^{bc}	1112.94 ± 115.05^{b}

462 ***Table 2. Gene expression levels of heavy metal ATPases (HMAs) in leaves and roots of A. thaliana.***
463 *Arabidopsis seedlings were either continuously grown under control conditions or Cu-deficiency or*
464 *nineteen-days-old plants were exposed to 5 µM $CdSO_4$ alone or in combination with additional $CuSO_4$*
465 *(0.5, 1 or 2 µM Cu extra in comparison to control Hoagland solution) for 72 h. Transcript levels were*
466 *calculated relative to the non-exposed plants (Control). Data are mean ± S.E. of 6 biological*
467 *replicates. Significant differences (P<0.05) after one-way ANOVA test and Tukey correction are*
468 *indicated with colour shading: red for reduction in comparison to control, green for induction in*
469 *comparison to control and yellow for induction in comparison to control but reduction in comparison*
470 *to 5 µM Cd.*

	Control	Cu deficiency	5 µM Cd	5 µM Cd + 0.5 µM Cu	5 µM Cd + 1 µM Cu	5 µM Cd + 2 µM Cu
Leaves						
Resolution	*1.00± 0.21*	*1.65± 0.84*	*0.51± 0.05*	*0.90± 0.30*	*1.41± 0.73*	*0.91± 0.23*
HMA2	1.00± 0.18	1.40± 0.04	0.85± 0.16	1.37± 0.08	0.98± 0.10	0.86± 0.08
HMA3	1.00± 0.24	1.71± 0.19	1.76± 0.17	2.00± 0.34	2.30± 0.41	2.46± 0.21
HMA4	1.00± 0.19	1.13± 0.05	1.19± 0.10	1.43± 0.12	1.10± 0.17	1.17± 0.14
HMA5	1.00± 0.11	1.01± 0.05	3.13± 0.39	2.15± 0.11	2.09± 0.31	2.26± 0.13
HMA7/RAN1	1.00± 0.04	1.14± 0.06	1.57± 0.19	1.29± 0.05	1.34± 0.07	1.37± 0.07
Roots						
Resolution	*1.00± 0.05*	*0.90± 0.02*	*0.94± 0.04*	*0.89± 0.03*	*0.99± 0.07*	*0.89± 0.03*
HMA2	1.00± 0.11	1.41± 0.04	0.71± 0.11	0.61± 0.03	0.31± 0.06	0.26± 0.04
HMA3	1.00± 0.16	1.13± 0.12	1.11± 0.08	1.01± 0.04	0.39± 0.07	0.38± 0.05
HMA4	1.00± 0.03	0.95± 0.03	1.59± 0.17	0.91± 0.04	1.23± 0.06	1.19± 0.05
HMA5	1.00± 0.03	0.98± 0.02	2.29± 0.13	1.62± 0.07	2.35± 0.25	2.15± 0.07
HMA7/RAN1	1.00± 0.03	1.06± 0.03	1.78± 0.09	1.18± 0.07	1.46± 0.15	1.30± 0.05

471

Table 3. Gene expression levels of oxidative stress hallmark genes in the leaves and roots of A. thaliana. *Arabidopsis seedlings were either continuously grown under control conditions or Cu-deficiency or nineteen-days-old plants were exposed to 5 µM $CdSO_4$ alone or in combination with additional $CuSO_4$ (0.5, 1 or 2 µM Cu extra in comparison to control Hoagland solution) for 72 h. Transcript levels were calculated relative to the non-exposed plants (Control). Data are mean ± S.E. of 6 biological replicates. Significant differences (P<0.05) after one-way ANOVA test and Tukey correction are indicated with colour shading: green for induction in comparison to control and yellow for induction in comparison to control but reduction in comparison to 5 µM Cd.*

	Control	Cu deficiency	5 µM Cd	5 µM Cd + 0.5 µM Cu	5 µM Cd + 1 µM Cu	5 µM Cd + 2 µM Cu
Leaves						
Resolution	*1.00 ± 0.26*	*2.01 ± 1.29*	*0.46 ± 0.05*	*0.62 ± 0.20*	*1.05 ± 0.48*	*0.71 ± 0.21*
UPOX	1.00 ± 0.06	1.20 ± 0.07	6.36 ± 1.28	4.59 ± 1.09	1.57 ± 0.45	1.28 ± 0.16
Defensin-like	1.00 ± 0.26	0.68 ± 0.14	27.22 ± 8.67	3.57 ± 0.91	2.38 ± 0.38	2.79 ± 0.42
AT1G19020	1.00 ± 0.47	0.32 ± 0.07	5.17 ± 1.61	2.73 ± 1.11	0.42 ± 0.08	0.49 ± 0.09
AT1G05340	1.00 ± 0.42	0.69 ± 0.13	11.58 ± 4.28	3.15 ± 1.16	0.74 ± 0.18	1.01 ± 0.21
TIR-class	1.00 ± 0.62	0.19 ± 0.07	1.53 ± 0.28	0.88 ± 0.29	0.28 ± 0.05	0.42 ± 0.13
Roots						
Resolution	*1.00 ± 0.06*	*0.88 ± 0.02*	*0.97 ± 0.03*	*0.88 ± 0.03*	*1.03 ± 0.06*	*0.90 ± 0.04*
UPOX	1.00 ± 0.08	1.38 ± 0.11	4.41 ± 0.69	3.19 ± 0.57	3.08 ± 0.32	5.82 ± 0.85
Defensin-like	1.00 ± 0.27	1.76 ± 0.39	211.98 ± 37.80	51.37 ± 6.81	473.36 ± 162.62	403.99 ± 68.46
AT1G19020	1.00 ± 0.12	1.65 ± 0.17	10.65 ± 1.54	5.38 ± 0.82	7.71 ± 1.59	8.76 ± 1.05
AT1G05340	1.00 ± 0.1	1.46 ± 0.07	2.34 ± 0.20	1.00 ± 0.11	1.36 ± 0.12	1.77 ± 0.10
TIR-class	1.00 ± 0.26	1.84 ± 0.13	20.50 ± 2.95	7.19 ± 1.56	21.52 ± 4.58	23.07 ± 2.69

Table 4. Elemental concentration ***in roots of A. thaliana wildtype and cad1-3 mutant plants.*** *Arabidopsis seedlings were either continuously grown under control conditions or nineteen-days-old plants were exposed to 5 µM $CdSO_4$ for 24 h and 72 h. Copper, Cd and potassium (K) concentrations in roots are presented in mg per kg dry weight (mg kg^{-1} DW). Data are mean ± S.E. of 4 biological replicates. Significant differences ($P<0.05$) after three-way ANOVA test and Tukey correction are indicated with different letters.*

		24 h		72 h	
		WT	***cad1-3***	**WT**	***cad1-3***
Cu	**Control**	14.75 ± 1.02^{ab}	12.11 ± 2.12^{ac}	7.48 ± 0.35^{c}	7.26 ± 0.29^{c}
	5 µM Cd	22.06 ± 2.66^{b}	39.18 ± 6.43^{d}	18.31 ± 1.69^{ab}	68.26 ± 4.15^{e}
Cd	**Control**	-	-	-	-
	5 µM Cd	654.32 ± 34.65^{a}	1710.73 ± 64.37^{b}	1104.88 ± 101.48^{a}	3692.53 ± 177.88^{c}
K	**Control**	49541.4 ± 2486.9^{ab}	50395.1 ± 1835.2^{a}	50685.8 ± 4199.70^{a}	49818.3 ± 1360.6^{ab}
	5 µM Cd	50114.2 ± 2900.0^{ab}	38928.5 ± 2143.2^{b}	47221.6 ± 2182.16^{ab}	14311.4 ± 599.2^{c}

488 ***Table 5. Gene expression levels of SPL7-regulated genes in roots of A. thaliana wildtype and cad1-3***
489 ***mutant plants.*** *Arabidopsis seedlings were either continuously grown under control conditions or*
490 *nineteen-days-old plants were exposed to 5 µM $CdSO_4$ for 24 h. Data are mean ± S.E. of at least 3*
491 *biological replicates relative to its own unexposed control (set at 1.00). Significant Cd-induced*
492 *differences (P<0.05) in expression within each genotype relative to the control after two-way ANOVA*
493 *test and Tukey correction are indicated in colour (upregulation, green; downregulation, red), while*
494 *differences between genotypes are indicated with asterisks. (ND, not detected after 40 cycles).*
495 *Abbreviations: FSD1, Fe superoxide dismutase1; COPT2, Cu transporter2; ZIP2, zinc-regulated*
496 *transporter2; CCH, Cu chaperone.*

		WT	*cad1-3*
Resolution	***Control***	*1.00 ± 0.03*	*1.00 ± 0.04*
	5 µM Cd	*0.94 ± 0.03*	*0.89 ± 0.04*
pri-miR397a	**Control**	1.00 ± 0.03	1.00 ± 0.03
	5 µM Cd	49.16 ± 13.52	0.88 ± 0.03*
pri-miR398b	**Control**	1.00 ± 0.10	1.00 ± 0.04
	5 µM Cd	2.06 ± 0.44	0.04 ± 0.00*
pri-miR398c	**Control**	1.00 ± 0.24	1.00 ± 0.17
	5 µM Cd	14.93 ± 3.86	0.08 ± 0.04*
pri-miR857	**Control**	1.00 ± 0.43	1.00 ± 0.66
	5 µM Cd	243.88 ± 43.04	ND
FSD1	**Control**	1.00 ± 0.21	1.00 ± 0.11
	5 µM Cd	6.93 ± 0.78	0.03 ± 0.00*
COPT2	**Control**	1.00 ± 0.28	1.00 ± 0.14
	5 µM Cd	7.11 ± 0.27	0.05 ± 0.02*
ZIP2	**Control**	1.00 ± 0.15	1.00 ± 0.08
	5 µM Cd	3.45 ± 0.06	0.02 ± 0.00*
CCH	**Control**	1.00 ± 0.08	1.00 ± 0.03
	5 µM Cd	2.19 ± 0.09	0.17 ± 0.01*

497

FIGURE LEGENDS

Figure 1. Fresh weight of leaves (black) and roots (white) of A. thaliana plants. *Arabidopsis seedlings were either continuously grown under control conditions or Cu-deficiency or nineteen-days-old control plants were exposed to 5 µM $CdSO_4$ alone or in combination with additional $CuSO_4$ (0.5, 1 or 2 µM Cu extra in comparison to control Hoagland solution) for 72 h. Data are mean ± S.E. of 6 biological replicates. Significant differences ($P<0.05$) after one-way ANOVA test and Tukey correction are indicated with different capital (roots) or small (leaves) letters.*

Figure 2. Cd translocation in A. thaliana. *Arabidopsis seedlings were either continuously grown under control conditions or Cu-deficiency or nineteen-days-old control plants were exposed to 5 µM $CdSO_4$ alone or in combination with additional $CuSO_4$ (0.5, 1 or 2 µM Cu extra in comparison to control Hoagland solution) for 72 h. The Cd translocation from roots to leaves was calculated as the concentration in the leaves relative to the concentration in the roots. Data are mean ± S.E. of 6 biological replicates. Significant differences ($P<0.05$) after one-way ANOVA test and Tukey correction are indicated with different letters.*

Figure 3. Gene expression levels of SPL7-regulated genes in the leaves and roots of A. thaliana. *Arabidopsis seedlings were either continuously grown under control conditions or Cu-deficiency or nineteen-days-old control plants were exposed to 5 µM $CdSO_4$ alone or in combination with additional $CuSO_4$ (0.5, 1 or 2 µM Cu extra in comparison to control Hoagland solution) for 72 h. Significant differences ($P<0.05$) after one-way ANOVA test and Tukey correction are indicated with colour shading: red for reduction in comparison to control, green for induction in comparison to control and yellow for induction in comparison to control but reduction in comparison to a single 5 µM Cd exposure. The relative fold changes are shown in Table S3. Abbreviations: FSD1, Fe superoxide dismutase1; CCH, Cu chaperone; COPT2, Cu transporter2; ZIP2, zinc-regulated transporter2.*

Figure 4. Fresh weight of leaves and roots of A. thaliana wildtype (black) and cad1-3 mutant (white) plants. *Arabidopsis seedlings were either continuously grown under control conditions or nineteen-days-old plants were exposed to 5 µM $CdSO_4$ for 24 h and 72 h. Data are mean ± S.E. of 4 biological replicates. Significant differences (P<0.05) after three-way ANOVA test and Tukey correction are indicated with different small (leaves) or capital (roots) letters.*

Figure 5. Cd translocation in A. thaliana wildtype (black) and cad1-3 mutant (white) plants. *Arabidopsis seedlings were either continuously grown under control conditions or nineteen-days-old plants were exposed to 5 µM $CdSO_4$ for 24 h and 72 h. The Cd translocation was calculated as the concentration in the shoots relative to the concentration in the roots. Data are mean ± S.E. of 7 biological replicates. Significant differences (P<0.05) after two-way ANOVA test and Tukey correction are indicated with different letters.*

Figure 6. Gene expression levels of HMA2 (A) and HMA4 (B) and HMA5 (C) in roots of A. thaliana wildtype and cad1-3 mutant plants. *Arabidopsis seedlings were either continuously grown under control conditions or nineteen-days-old plants were exposed to 5 µM $CdSO_4$ for 24 h. Data are mean ± S.E. of at least 3 biological replicates relative to its own unexposed control (set at 1.00). Significant Cd-induced differences (P<0.05) in expression within each genotype relative to the control after two-way ANOVA test and Tukey correction are indicated with asterisks.*

Figure 7. Schematic model illustrating the cellular working mechanism explaining the Cd-induced activation of the SPL7-dependent Cu deficiency response in roots of A. thaliana. *In wildtype plants (indicated in orange), exposure to Cd results in high Cd and Cu concentrations in the roots which induces PC synthesis for Cd detoxification. Upon this process, also Cu is partly sequestered resulting in cellular Cu deficiency by which the TF SPL7 is activated in an unknown manner. SPL7 induces transcription of several genes involved in Cu uptake and Cu reallocation. This response is called the Cu deficiency response and is needed for basal Cd tolerance. By inducing the Cu deficiency response, the metal transporter ZIP2 is also upregulated, stimulating Cu uptake as well as Cd uptake. In*

553 *addition, Cd exposure also increases transcript levels of the xylem loading heavy metal transporters*
554 *HMA2 and HMA4 resulting in a stimulated Cd translocation to the shoots, where subsequently the Cu*
555 *deficiency response is induced. When extra Cu is supplied to Cd-exposed plants (indicated in green),*
556 *the Cu* concentration *increases and the Cd* concentration *decreases, concomitantly the Cu deficiency*
557 *response diminishes. Therefore we suggest that it is mainly the amount of Cd, which determines the*
558 *level of PC production and as such the partial sequestration of Cu and hence the induction of the Cu*
559 *deficiency response. To further support this mechanism, the Cu deficiency response is not induced in*
560 *Cd-exposed cad1-3 mutant (indicated in purple), which is unable to produce phytochelatins (PCs).*
561 *Furthermore, adding extra Cu to Cd-exposed plants downregulates transcription of HMA2 and HMA4*
562 *resulting in a decreased Cd translocation to the shoots and consistently a reduced Cu deficiency*
563 *response in the leaves. Similarly in the cad1-3 mutant, Cd exposure decreased expression levels*
564 *HMA2 and HMA4 leading to a decreased Cd translocation to the shoots.*
565

SUPPORTING INFORMATION

Table S1. Forward and reverse primers used to determine gene expression levels via quantitative real-time PCR*. E-E-jn, exon-exon junction; E-I-b, exon-intron boundary; YLS, yellow-leaf-specific; UBQ10, ubiquitin; ACT, actin ; EF, elongation factor; FSD1, Fe superoxide dismutase.*

Table S2. Quantitative real-time PCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin et al., 2009.

Table S3. Gene expression levels of SPL7-regulated genes in the leaves and roots of A. thaliana. *Nineteen-days-old plants were further grown under control conditions or $CuSO_4$ deficiency, or were exposed to 5 µM $CdSO_4$ and supplemented with extra $CuSO_4$ (0, 0.5, 1 or 2 µM Cu extra compared to control Hoagland solution) for 72 h. Significant differences ($P<0.05$) after one-way ANOVA test and Tukey correction are indicated with different letters.*