Made available by Hasselt University Library in https://documentserver.uhasselt.be

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

Peer-reviewed author version

GIELEN, Heidi; VANGRONSVELD, Jaco & CUYPERS, Ann (2017) Cd-induced Cu deficiency responses in Arabidopsis thaliana: are phytochelatins involved?. In: PLANT CELL AND ENVIRONMENT, 40(3), p. 390-400.

DOI: 10.1111/pce.12876
Handle: http://hdl.handle.net/1942/24104

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

Running title: Cd-induced Cu deficiency responses

Heidi Gielen[1], Jaco Vangronsveld[1], Ann Cuypers[1*]

[1]Environmental Biology, Centre for Environmental Sciences, Hasselt University, Agoralaan Building D, B-3590 Diepenbeek, Belgium

[*]corresponding author: Ann Cuypers, ann.cuypers@uhasselt.be

Address: Hasselt University – Campus Diepenbeek, Agoralaan Building D, B-3590 Diepenbeek, Belgium, tel +32(0)11 26 83 26, fax +32(0)11 26 82 29

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

31 **INTRODUCTION**

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

36 Since Cd is non-essential, plants do not possess specific transporters for this metal. Instead, Cd enters
37 root cells through transporters of essential nutrients like the nonspecific transporters ZIP/IRT1 (zinc-
38 regulated transporter/iron-regulated transporter) and calcium channels (Clemens, 2006; Verbruggen et
39 al., 2009). Once inside the cell, the distribution of Cd to cell organelles and throughout the plant is
40 possible via several transporters like heavy metal ATPases (HMAs). In *Arabidopsis thaliana*, it was
41 demonstrated that HMA3 is located in the tonoplast transporting Cd into the vacuole (Morel et al.,
42 2009), while HMA2 and HMA4 can load Cd into the xylem for long-distance transport (Wong &
43 Cobbett, 2009).

44 Since Cd will be transported throughout the plant, it can interfere with several cellular processes such
45 as photosynthesis and respiration (DalCorso et al., 2008). Furthermore, Cd indirectly causes the
46 generation of reactive oxygen species (ROS) resulting in an oxidative challenge (Cuypers et al., 2012;
47 Sharma & Dietz, 2009). The presence of an antioxidative defence network, consisting of metabolites
48 [*e.g.* GSH and ascorbate (AsA)] and enzymes [*e.g.* superoxide dismutase (SOD), peroxidase (POD)
49 and catalase (CAT)], provides plants with the ability to cope with this Cd-induced oxidative stress.
50 Furthermore, Cd can disturb the homeostasis of essential nutrients through competition with their
51 uptake and with their binding sites in metalloproteins (DalCorso et al., 2008; Sharma & Dietz, 2009).
52 For example, it was demonstrated that Cd disturbed iron (Fe) levels in shoots and roots and this was
53 molecularly supported by increased transcript levels of *IRT1* and *FRO2* (ferric reductase oxidase) (Xu
54 et al., 2015; Yoshihara et al., 2006). It is also proven that Cd interferes with Cu homeostasis, inducing
55 a Cu deficiency response dependent on SPL7 (SQUAMOSA promoter binding protein like7), the
56 master regulator transcription factor (TF) of Cu homeostasis (Gayomba et al., 2013; Gielen et al.,
57 2016; Yamasaki et al., 2009). In this Cu deficiency response on the one hand, several Cu transporters,
58 like COPT2 and ZIP2, are upregulated to increase Cu uptake. On the other hand, the so-called cupro-

59 miRNAs targeting transcripts of Cu-containing proteins are increased to reallocate Cu to essential
60 proteins. Gielen *et al.* (2016) suggested that Cd possibly provokes Cu deficiency, thereby activating
61 SPL7 and subsequently inducing the Cu deficiency response.
62 Therefore, it is important that plants possess defence strategies to limit free Cd. Once inside the cell,
63 Cd is detoxified and sequestered to the least sensitive parts in the cell (*e.g.* vacuole) by binding to
64 chelators like glutathione (GSH) and phytochelatins (PCs). Glutathione (γ-glu-cys-gly) has a thiol
65 group, a favoured ligand of Cd, and polymerization of 2-11 moieties from GSH molecules by PC
66 synthase (PCS) results in the formation of PCs. The need for this chelating capacity is high in Cd-
67 exposed plants and therefore these plants increase their GSH and PC levels as demonstrated in several
68 studies (Jozefczak et al., 2014; Sofo et al., 2013). Furthermore, *cad2* mutants with reduced GSH and
69 PC levels and *cad1* mutants lacking PCs are hypersensitive to Cd (Cobbett, 2000; Howden et al.,
70 1995; Jozefczak et al., 2015; Sofo et al., 2013). Therefore, this chelating capacity is very important for
71 Cd tolerance and detoxification.
72 The aim of this study is to explore how Cd provokes Cu deficiency in *A. thaliana* and subsequently
73 induces the Cu deficiency response. Therefore, we exposed wildtype plants to a Cd containing nutrient
74 solution supplemented with extra Cu and monitored the impact of this exposure on metal
75 concentration and transcript levels of SPL7-regulated genes and heavy metal transporters.
76 Furthermore, we investigated the involvement of PC production in the Cd-induced Cu deficiency
77 response by using a *cad1-3* mutant that is deficient in PC-synthase activity and thus the formation of
78 PCs.
79

80 **MATERIALS AND METHODS**

81 **Plant culture, exposure and harvest**

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

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

91

92 **Gene expression analysis**

93 Frozen tissues (50-75 mg FW) were disrupted using two stainless steel beads and the Retsch Mixer
94 Mill MM400 (Retsch, Belgium) under frozen conditions. Total RNA was extracted using the Mirvana
95 Kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions (Total RNA
96 isolation). cDNA samples have an RNA input of 1 µg that was DNase treated (Turbo DNA-freeTM
97 kit, Life Technologies) and reverse transcribed using the PrimeScript RT reagent Kit (Perfect Real
98 Time, Takara Bio inc., Westburg, The Netherlands) according to manufacturer's instructions. A 10-
99 fold dilution of cDNA was made using 1/10 diluted TE buffer (1 mM Tris-HCL, 0.1 mM EDTA, pH
100 8.0, Sigma-Aldrich, Belgium) and stored at -20°C.

101 Quantitative PCR analysis was performed with the 7500HT Fast Real-Time PCR System (Life
102 Technologies). Primers were designed using primer express 2.0 and a BLAST was performed
103 (http://www.arabidopsis.org/Blast/index.jsp) to check specificity (Table S1). A two-fold dilution series
104 of a pooled sample (all cDNAs of the experiment) was used to create a standard curve for the
105 evaluation of primer efficiencies that were accepted when they were within a range between 80% and
106 100% (measured over 6 dilution points). PCR amplifications were performed at universal cycling
107 conditions and contained Fast SYBR Green Master Mix, 300 nM of a gene-specific forward and
108 reverse primer, and 2 µl of the diluted cDNA in a final volume of 10 µl.

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

111 All relative expression levels were calculated as $2^{-\Delta Cq}$ and normalized by the geometric average of $2^{-\Delta Cq}$
112 values of minimum three reference genes selected by the GrayNorm algorithm (Remans et al.,
113 2014). Data of treatment effects are expressed relative to the control of its own genotype set at 1.00.
114 For some genes expression was not detected after 40 cycles under control conditions. In that case,

115 cyclic threshold was set at 40 for the control samples and thereby treatment effects could be calculated
116 and expressed relative to the control. The calculated and represented treatment effects are therefore
117 minimal fold changes when expression was undetermined after 40 cycles under control conditions. All
118 details of the workflow according to the Minimum Information for publication of Quantitative real-
119 time PCR Experiments (MIQE) guidelines as described by Bustin *et al.* (2009) are shown in Table S2.
120

121 **Element analysis**

122 Fresh root samples (175-400 mg FW) were washed for 15 min with 10 mM $Pb(NO_3)_2$ at 4°C and
123 rinsed with distilled water, while leaf samples (400-1000 mg FW) were only rinsed with distilled
124 water. Samples were oven-dried (60°C for 3 weeks), weighed, digested with 70% HNO_3 in a heat
125 block and dissolved in 5 ml of 2% HCl. Copper and Cd concentrations were measured via inductively
126 coupled plasma-atomic emission spectrometry (ICP-OES, Agilent Technologies, 700 series, Belgium).
127 Blanks (only HNO_3) and standard references (NIST Spinach 1570a) were included.
128

129 **Statistical analysis**

130 Statistical analysis was performed using RStudio (version 0.99.485; R Foundation for Statistical
131 Computing, Vienna, Austria). Normal distribution of the datasets was tested using the Shapiro-Wilk
132 test and homoscedasticity was evaluated with the Bartlett's test. If necessary, transformations of the
133 datasets were applied. Gene expression data were always log transformed. Significant differences were
134 determined using ANOVA test and Tukey correction. If the assumption of normality was not fulfilled,
135 a non-parametrical ANOVA test (Kruskal-Wallis) and correction with pairwise Wilcoxon rank sum
136 test was applied.
137

138 **RESULTS**

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

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

143 under Cu deficiency or were exposed to 5 µM Cd alone or supplemented with extra Cu (0.5, 1 or 2
144 µM) for 72 h.
145
146 ***Plant fresh weight decreases with increasing Cu concentration***
147 The fresh weight (FW) of leaves and roots was determined in plants grown under control conditions
148 and after 72 h exposure to 5 µM Cd alone or supplemented with extra Cu and in plants grown under
149 Cu deficiency from germination on (Figure 1). Copper deficient plants showed no differences in leaf
150 and root FW in comparison to control plants. Exposure to 5 µM Cd significantly decreased root FW,
151 yet not leaf FW. However, additional Cu in combination with 5 µM Cd decreased leaf and root FW
152 with increasing Cu, although no differences between the addition of 1 and 2 µM Cu extra were
153 observed.
154
155 ***Additional Cu provokes alterations in Cu and Cd concentration***
156 Plants grown under Cu deficiency had significantly lower Cu levels in both roots and leaves (Table 1).
157 Significantly lower Cu levels were also found in the leaves after exposure to Cd as well as after
158 exposure to Cd supplemented with 0.5 µM Cu. However, adding higher amounts of Cu (1 and 2 µM
159 Cu) together with Cd did not lead to leaf Cu concentrations different from the control plants (Table 1).
160 On the contrary, in the roots of Cd-exposed plants, Cu concentration was significantly increased and
161 adding extra Cu dose-dependently increased Cu levels even more. In both leaves and roots, the Cd
162 concentration was significantly diminished in plants exposed to Cd supplemented with extra Cu in
163 comparison to plants exposed to Cd alone (Table 1).
164
165 The translocation of Cd from roots to shoots was significantly reduced in plants exposed to 5 µM Cd
166 supplemented with 1 or 2 µM Cu in comparison to plants exposed to Cd only (Figure 2).
167
168 ***Gene expression levels of SPL7-dependent genes***
169 Since the transcription factor SPL7 is involved in the response to Cd exposure and in Cu homeostasis
170 (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*

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

205

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

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

209

210 ***Cd-induced effects on growth***

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

216

217 ***Cd-induced effects on elemental concentration***

218 In wildtype plants, the Cu concentration in the roots was significantly increased after Cd exposure for
219 72 h, while in the *cad1-3* mutant plants Cu concentration was already significantly increased after 24 h
220 Cd exposure (Table 4). Furthermore, the Cu concentration in the roots was significantly higher in Cd-
221 exposed *cad1-3* mutant plants in comparison to wildtype plants. Similarly, Cd-exposed *cad1-3* mutant
222 plants had a higher Cd concentration in the roots then wildtype plants (Table 4). Potassium (K)
223 concentration was significantly decreased in the roots of the Cd-exposed *cad1-3* mutant plants in
224 comparison to *cad1-3* control plants, while K concentration didn't change in wildtype plants (Table 4).
225 Furthermore, the Cd concentration in the shoots (data not shown) and the Cd translocation from roots

226 to shoots was significantly lower in *cad1-3* mutant plants in comparison to wildtype plants when
227 plants were exposed to 5 µM Cd for 24 h and 72 h (Figure 5).
228
229 ***Opposite Cd-induced gene expressions in the cad1-3 mutant in comparison to wildtype plants***
230 Phytochelatins are already important early on in roots upon Cd exposure (Jozefczak et al., 2014). To
231 unravel a possible link between PCs and the Cd-induced Cu deficiency response, we focused on
232 alteration of gene expression in roots. Since *cad1-3* mutant plants severely suffered from exposure to
233 Cd for 72 h (Figure 4 and Table 4), gene expression analysis was performed on plants exposed to Cd
234 for 24 h. In wildtype plants, the transcript levels of SPL7-regulated genes were significantly
235 upregulated after exposure to 5 µM Cd (Table 5) similar to what is seen in Figure 1. In contrast to the
236 wildtype, exposure to 5 µM Cd in the *cad1-3* mutant did not alter these expression levels or even led
237 to a downregulation of these transcripts (Table 5).
238
239 ***Changes in gene expression of Cd transporters***
240 The expressions of both *HMA2* and *HMA4* were significantly lower in Cd-exposed *cad1-3* mutant
241 plants in comparison to their control, while *the* expression was not significantly altered in wildtype
242 plants (Figure 6A and B). Transcript levels of *HMA5* were significantly increased in Cd-exposed WT
243 plants in comparison to WT control plants, while no differences were demonstrated for *cad1-3* mutant
244 plants (Figure 6C).
245
246 **DISCUSSION**
247 A major mechanism by which Cd exerts its toxicity is the disturbance of the homeostasis of essential
248 elements. Previous results have indicated a Cd-induced disturbance of the Cu homeostasis in *A.*
249 *thaliana* (Gayomba et al., 2013; Gielen et al., 2016). Cadmium exposure changed the Cu concentration
250 in leaves and roots and this was accompanied by a Cu deficiency response at transcriptional level. It
251 was demonstrated that this Cd-induced Cu deficiency response is dependent on the transcription factor
252 SPL7 (Gayomba et al., 2013; Gielen et al., 2016), the master regulator of Cu homeostasis (Yamasaki
253 et al., 2009). In Cu limited conditions, SPL7 activates this response, which includes on the one hand

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

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

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

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

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

310 Consequently growth is reduced in a dose-dependent manner when plants are exposed to Cd with extra
311 Cu, which is demonstrated by the decreased fresh weights of leaves and roots (Figure 1). At the
312 cellular level however, leaves of these exposed plants handle this combined metal stress quite well
313 since oxidative stress levels diminished in comparison to single Cd-exposed plants (Table 3). On the
314 contrary, roots of plants exposed to Cd in combination with extra Cu appeared to suffer more than in
315 single Cd-exposed plants. In general, combined exposures significantly increased transcript levels of
316 the oxidative stress hallmark genes in the roots in comparison to control plants (Table 3). However, a
317 differentiation should be made between Cd-exposed plants supplemented with on the one hand 0.5 μM
318 Cu and on the other hand supplemented with 1 or 2 μM Cu. The addition of 0.5 μM Cu to Cd-exposed
319 plants decreased transcript levels of the oxidative stress hallmark genes in the roots in comparison with
320 plants exposed to Cd alone (Table 3), which indicates that the cellular oxidation state had improved. In
321 contrast, 1 or 2 μM Cu supplemented to Cd-exposed plants showed no difference in transcript levels of
322 these oxidative stress hallmark genes in comparison to plant roots exposed to Cd alone, and even an
323 increasing trend was seen for *Defensin-like* and *TIR-class* transcript levels (Table 3). Therefore, it can
324 be concluded that adding a low amount of Cu to Cd-exposed plants improves the cellular oxidation
325 state of the plants, while higher amounts of Cu evoked Cu toxicity in plant roots.
326 Although the Cu deficiency response disappeared in the leaves and diminished in the roots when extra
327 Cu was supplied to Cd-exposed plants, Cd was still present in leaves and roots, though to a lesser
328 extent. An important defence strategy of plants against Cd is the complexation and sequestration
329 through PCs. The preferred ligands of Cd and Cu are thiols (SH-groups), which are multiple times
330 present in PCs. It is demonstrated that Cd increases PC levels in *A. thaliana under similar conditions*
331 *as our experiments* (Jozefczak et al., 2014) and that *cad1-3* mutants lacking PCs are hypersensitive to
332 Cd (Howden et al., 1995; Sofo et al., 2013). Besides, exposure to excess Cu, from which it is known to
333 be detoxified by metallothioneins (Cobbett & Goldsbrough, 2002; Guo et al., 2008), also resulted in
334 increased PC levels in *Arabidopsis* seedlings (Sofo et al., 2013). From that, we hypothesized that the
335 PCs induced by Cd can also complex Cu resulting in a Cu deficiency response, though Cu
336 concentration is strictly not limited in the plant. To investigate this hypothesis, a *cad1-3* mutant was
337 used which is defective in PC synthesis (G/C nucleotide exchange in the *PCS1* locus). Cadmium-

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

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

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

373

374 **ACKNOWLEDGEMENTS**

375 This work was supported by Hasselt University by a PhD grant for Heidi Gielen and through the
376 Methusalem project [08M03VGRJ]. We thank Ann Wijgaerts and Carine Put for their skilful technical
377 assistance and we are grateful to Prof Dr Luis E. Hernandez to donate *cad1-3* mutant seeds.

378

379 **REFERENCES**

380 Andrés-Colas N., Sancenon V., Rodriguez-Navarro S., Mayo S., Thiele D. J., Ecker J. R., …
381 Penarrubia L. (2006). The Arabidopsis heavy metal P-type ATPase HMA5 interacts with
382 metallochaperones and functions in copper detoxification of roots. *The Plant Journal* 45, 225–
383 236.

384 Bustin S. A., Benes V., Garson J. A., Hellemans J., Huggett J., Kubista M., … Wittwer C. T. (2009).
385 The MIQE guidelines:Minimum Information for publication of quantitative real-time PCR
386 experiments. *Clinical Chemistry* 55, 611–622.

387 Clemens S. (2006). Toxic metal accumulation, responses to exposure and mechanisms of tolerance
388 in plants. *Biochimie* 88, 1707–1719.

389 Cobbett C., & Goldsbrough P. (2002). Phytochelatins and metallothioneins: roles in heavy metal
390 detoxification and homeostasis. *Annual Review of Plant Biology* 53, 159–82.

391 Cobbett C. S. (2000). Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology*
392 123, 825–832.

393 Cuypers A., Keunen E., Bohler S., Jozefczak M., Opdenakker K., Gielen H., … Remans T. (2012).

394 Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus
395 signalling. In D. K. G. Gupta & L. M. Sandalio (Eds.), *Metal Toxicity in Plants: Perception,*
396 *Signaling and Remediation* (pp. 65–90). Springer-Verlag GmbH, Berlin, Heidelberg.
397 DalCorso G., Farinati S., Maistri S., & Furini A. (2008). How plants cope with cadmium: staking all
398 on metabolism and gene expression. *Journal of Integrative Plant Biology* 50, 1268–80.
399 Gadjev I., Vanderauwera S., Gechev T. S., Laloi C., Minkov I. N., Shulaev V., … Breusegem F. Van.
400 (2006). Transcriptomic footprints disclose specificity of reactive oxygen species signaling in
401 Arabidopsis. *Plant Physiology* 141, 436–445.
402 Gayomba S. R., Jung H., Yan J., Danku J., Rutzke M. A., Bernal M., … Vatamaniuk O. K. (2013).
403 The CTR/COPT-dependent copper uptake and SPL7-dependent copper deficiency responses are
404 required for basal cadmium tolerance in A. thaliana. *Metallomics* 5, 1262–75.
405 Gielen H., Remans T., Vangronsveld J., & Cuypers A. (2016). Toxicity responses of Cu and Cd: the
406 involvement of miRNAs and the transcription factor SPL7. *BMC Plant Biology* 16, 145.
407 Guo W.-J., Meetam M., & Goldsbrough P. B. (2008). Examining the specific contributions of
408 individual Arabidopsis metallothioneins to copper distribution and metal tolerance. *Plant*
409 *Physiology* 146, 1697–1706.
410 Howden R., Goldsbrough P. B., Andersen C. R., & Cobbett C. S. (1995). Cadmium-sensitive, cad1
411 mutants of Arabidopsis thaliana are phytochelatin deficient. *Plant Physiology* 107, 1059–66.
412 Jozefczak M., Bohler S., Schat H., Horemans N., Guisez Y., Remans T., … Cuypers A. (2015). Both
413 the concentration and redox state of glutathione and ascorbate influence the sensitivity of
414 arabidopsis to cadmium. *Annals of Botany* 601–612.
415 Jozefczak M., Keunen E., Schat H., Bliek M., Hernández L. E., Carleer R., … Cuypers A. (2014).
416 Differential response of Arabidopsis leaves and roots to cadmium: Glutathione-related chelating
417 capacity vs antioxidant capacity. *Plant Physiology and Biochemistry* 83, 1–9.
418 Keunen E., Jozefczak M., Remans T., Vangronsveld J., & Cuypers A. (2013). Alternative respiration
419 as a primary defence during cadmium-induced mitochondrial oxidative challenge in Arabidopsis
420 thaliana. *Environmental and Experimental Botany* 91, 63–73.
421 Morel M., Crouzet J., Gravot A., Auroy P., Leonhardt N., Vavasseur A., & Richaud P. (2009).

422 AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in Arabidopsis. *Plant*
423 *Physiology* 149, 894–904.
424 Remans T., Keunen E., Bex G. J., Smeets K., Vangronsveld J., & Cuypers A. (2014). Reliable gene
425 expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the
426 uncertainty in data accuracy. *The Plant Cell* 26, 3829–3837.
427 Sharma S. S., & Dietz K.-J. (2009). The relationship between metal toxicity and cellular redox
428 imbalance. *Trends in Plant Science* 14, 43–50.
429 Smeets K., Ruytinx J., Van Belleghem F., Semane B., Lin D., Vangronsveld J., & Cuypers A. (2008).
430 Critical evaluation and statistical validation of a hydroponic culture system for Arabidopsis
431 thaliana. *Plant Physiology and Biochemistry* 46, 212–218.
432 Sofo A., Vitti A., Nuzzaci M., Tataranni G., Scopa A., Vangronsveld J., … Sanità di Toppi L. (2013).
433 Correlation between hormonal homeostasis and morphogenic responses in Arabidopsis thaliana
434 seedlings growing in a Cd/Cu/Zn multi-pollution context. *Physiologia Plantarum* 149, 487–498.
435 Verbruggen N., Hermans C., & Schat H. (2009). Mechanisms to cope with arsenic or cadmium excess
436 in plants. *Current Opinion in Plant Biology* 12, 364–72.
437 Verret F., Gravot A., Auroy P., Leonhardt N., David P., Nussaume L., … Richaud P. (2004).
438 Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant
439 metal tolerance. *FEBS Letters* 576, 306–312.
440 Wong C. K. E., & Cobbett C. S. (2009). HMA P-type ATPases are the major mechanism for root-to-
441 shoot Cd translocation in Arabidopsis thaliana. *New Phytologist* 181, 71–78.
442 Xu S. S., Lin S. Z., & Lai Z. X. (2015). Cadmium impairs iron homeostasis in Arabidopsis thaliana by
443 increasing the polysaccharide contents and the iron-binding capacity of root cell walls. *Plant and*
444 *Soil* 392, 71–85.
445 Yamasaki H., Hayashi M., Fukazawa M., Kobayashi Y., & Shikanai T. (2009). SQUAMOSA
446 Promoter Binding Protein-Like7 is a central regulator for copper homeostasis in Arabidopsis.
447 *The Plant Cell* 21, 347–61.
448 Yoshihara T., Hodoshima H., Miyano Y., Shoji K., Shimada H., & Goto F. (2006). Cadmium
449 inducible Fe deficiency responses observed from macro and molecular views in tobacco plants.

450 *Plant Cell Reports* 25, 365–73.

451

452 **TABLES**

453 ***Table 1. Copper and Cd concentrations in leaves and roots of A. thaliana.*** *Arabidopsis seedlings*
454 *were either continuously grown under control conditions or Cu-deficiency or nineteen-days-old plants*
455 *were exposed to 5 µM $CdSO_4$ alone or in combination with additional $CuSO_4$ (0.5, 1 or 2 µM Cu extra*
456 *in comparison to control Hoagland solution) for 72 h. Copper and Cd* concentration*s are presented in*
457 *mg per kg dry weight (mg kg^{-1} DW). Data are mean ± S.E. of 6 biological replicates. Significant*
458 *differences (P<0.05) after one-way ANOVA test and Tukey correction (leaves) or after a non-*
459 *parametrical ANOVA test (Kruskal-Wallis) and correction with pairwise Wilcoxon rank sum test*
460 *(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]

461

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

472 ***Table 3. Gene expression levels of oxidative stress hallmark genes in the leaves and roots of A.***
473 ***thaliana.*** *Arabidopsis seedlings were either continuously grown under control conditions or Cu-*
474 *deficiency or nineteen-days-old plants were exposed to 5 µM $CdSO_4$ alone or in combination with*
475 *additional $CuSO_4$ (0.5, 1 or 2 µM Cu extra in comparison to control Hoagland solution) for 72 h.*
476 *Transcript levels were calculated relative to the non-exposed plants (Control). Data are mean ± S.E.*
477 *of 6 biological replicates. Significant differences ($P<0.05$) after one-way ANOVA test and Tukey*
478 *correction are indicated with colour shading: green for induction in comparison to control and yellow*
479 *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

480

481 ***Table 4. Elemental*** concentration ***in roots of A. thaliana wildtype and cad1-3 mutant plants.***
482 *Arabidopsis seedlings were either continuously grown under control conditions or nineteen-days-old*
483 *plants were exposed to 5 µM $CdSO_4$ for 24 h and 72 h. Copper, Cd and potassium (K) concentrations*
484 *in roots are presented in mg per kg dry weight (mg kg^{-1} DW). Data are mean ± S.E. of 4 biological*
485 *replicates. Significant differences ($P<0.05$) after three-way ANOVA test and Tukey correction are*
486 *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]

487

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

498 **FIGURE LEGENDS**

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

505

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

513

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

524

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

530

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

537

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

544

545 ***Figure 7. Schematic model illustrating the cellular working mechanism explaining the Cd-induced***
546 ***activation of the SPL7-dependent Cu deficiency response in roots of A. thaliana.*** *In wildtype plants*
547 *(indicated in orange), exposure to Cd results in high Cd and Cu concentrations in the roots which*
548 *induces PC synthesis for Cd detoxification. Upon this process, also Cu is partly sequestered resulting*
549 *in cellular Cu deficiency by which the TF SPL7 is activated in an unknown manner. SPL7 induces*
550 *transcription of several genes involved in Cu uptake and Cu reallocation. This response is called the*
551 *Cu deficiency response and is needed for basal Cd tolerance. By inducing the Cu deficiency response,*
552 *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

566 **SUPPORTING INFORMATION**

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

570

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

574

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