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1 **Original paper**

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3 **Accession-specific life strategies affect responses in leaves of**

4 ***Arabidopsis thaliana* plants exposed to excess Cu and Cd**

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31 **Summary**

32 The natural accession Columbia (Col-0) is considered as the reference genome of the model
33 plant *Arabidopsis thaliana*. Nonetheless, Col-0 plants are more sensitive to excess copper (Cu)
34 and cadmium (Cd) than other widely used accessions such as Wassilewskija (Ws) plants. In the
35 current study, this accession-specific metal sensitivity is further explored by comparing the
36 responses in leaves of Col-0 and Ws plants exposed to excess Cu and Cd.

37 Our results suggest that different life strategies favored by both accessions under physiological
38 conditions affect their response to metal exposure. While Col-0 plants mainly invest in metal
39 detoxification, Ws plants center on nutrient homeostasis. In particular, the higher expression of
40 genes related to Cu homeostasis genes in non-exposed conditions indicates that Ws plants possess
41 a constitutively efficient metal homeostasis. On the other hand, oxidative stress-related MAPK
42 signaling appears to be boosted in leaves of Col-0 plants exposed to excess Cu. Furthermore, the
43 upregulation of the glutathione (GSH) biosynthesis *GSH2* gene and the increased GSH
44 concentration after Cd exposure suggest the activation of detoxification mechanisms, such as
45 phytochelatin production, to counteract the more severe Cd-induced oxidative stress in leaves of
46 Col-0 plants. Exposure to Cd also led to a more pronounced ethylene signaling response in leaves
47 of Col-0 as compared to Ws plants, which could be related to Cd-induced GSH metabolism. In
48 conclusion, accession-specific life strategies clearly affect the way in which leaves of *A. thaliana*
49 plants cope with excess Cu and Cd.

50

51 **Keywords:** *Arabidopsis thaliana*; Natural accessions; Leaves; Copper; Cadmium

52 1. Introduction

53 Anthropogenic activities have a worldwide impact on soil elemental composition. In turn, this
54 represents an obstacle to normal plant development. For example, plant survival is commonly
55 affected in metal-enriched environments where excessive concentrations of plant-available metals
56 cause phytotoxicity and inhibit plant growth. However, this toxicity response is highly dependent
57 on the chemical properties of the metal involved. For example, excess copper (Cu) and cadmium
58 (Cd) disturb normal plant metabolism in different ways (Cuypers et al., 2012; Mourato et al.,
59 2012). Copper is a redox-active essential micronutrient that inhibits enzyme functioning and
60 interferes with essential cellular processes when present in excess (Cuypers et al., 2011; Gielen et
61 al., 2016, 2017; Lequeux et al., 2010; Yruela, 2005, 2009). It also directly induces oxidative
62 stress by catalyzing the formation of reactive oxygen species (ROS) through Fenton and Haber-
63 Weiss reactions (Drażkiewicz et al., 2004). Alternatively, Cd is a highly phytotoxic non-essential
64 element affecting plant growth even when available in low concentrations (Jozefczak et al., 2014;
65 Keunen et al., 2011, 2013; Park et al., 2012, Schellingen et al., 2015b, Wong and Cobbett, 2009).
66 It triggers oxidative stress in an indirect way by interfering with the cellular metabolism and
67 antioxidative mechanisms (Cuypers et al., 2011; Jozefczak et al., 2014, 2015; Schellingen et al.,
68 2015a).

69 *Arabidopsis thaliana* is a well-established model plant in molecular and genetic studies.
70 Notwithstanding several natural accessions existing, the Columbia (Col-0) accession is generally
71 acknowledged as the reference genome (Weigel, 2012) and is the subject of intensive study.
72 Nonetheless, exploring and comparing the responses of different natural accessions can provide
73 new insights into our current knowledge, for example on stress responses induced by excess soil
74 metal concentrations. Albeit limited, some comparative studies described differences in metal

75 sensitivity between different *A. thaliana* accessions. Indeed, Col-0 plants have been demonstrated
76 to be more sensitive to excess Cu and Cd than plants of other widely used *Arabidopsis* accessions
77 such as Wassilewskija (Ws) or Landsberg erecta (*Laer*) (Amaral dos Reis et al., submitted;
78 Murphy and Taiz, 1995a, 1995b, 1997; Park et al., 2012; Schiavon et al., 2007).

79 In their study using 10 different *A. thaliana* accessions, Murphy and Taiz (1995a) described
80 Col-0 as the accession showing the lowest constitutive Cu tolerance. Furthermore, they reported
81 that Ws plants showed an acclimation response to Cu, resulting in significant levels of inducible
82 Cu tolerance, which was later related to higher basal levels of non-protein thiols and glutathione
83 (GSH) (Murphy and Taiz, 1995b). In subsequent studies, the authors correlated this accession-
84 specific Cu tolerance to a distinctive regulation of the Cu-chelating metallothionein 2 (*MT2*) gene
85 (Murphy and Taiz, 1995b) and to differences in the ability to reverse Cu-induced potassium
86 leakage (Murphy and Taiz, 1997). Schiavon et al. (2007) also explored this accession-specific
87 variation in Cu sensitivity. Since the less sensitive Ws plants accumulated more Cu in the roots
88 and shoots than the more sensitive Col-0 plants, they reasoned that Cu exclusion is not the main
89 tolerance mechanism of the former accession. Alternatively, they hypothesized that a higher Cu
90 sensitivity is a manifestation of cation imbalance in the cell, ultimately reflecting Cu-induced
91 nutrient deficiency (Schiavon et al., 2007). Park et al. (2012) described Col-0 plants to be more
92 sensitive to Cd than Ws plants, potentially related to the non-functioning of heavy metal ATPase
93 3 (*HMA3*) in Col-0 plants and a differential expression of other *HMA* genes in both accessions.
94 Considering differential localization and expression patterns, it was hypothesized that the
95 combined action of the vacuolar transporter *HMA3* and the plasma membrane transporters
96 *HMA2* and *HMA4* is relevant to detoxify Cd in plants with a lower Cd sensitivity. While *HMA3*
97 results in Cd sequestration in root vacuoles, *HMA2* and *HMA4* limit root-to-shoot Cd

98 translocation, ultimately preventing competition between different elements and nutrient
99 deficiency symptoms in the shoots of Ws plants (Park et al., 2012).

100 In addition, our recent study further supports Ws plants being less sensitive to excess Cu and
101 Cd than Col-0 plants after comparing metal-induced responses in roots (Amaral dos Reis et al.,
102 submitted). More specifically, Ws plants were better able to respond to the alterations in Cu
103 homeostasis induced by both Cu and Cd exposure. Recently, it was shown that Cd exposure
104 induces Cu deficiency-like responses in Col-0 plants (Gielen et al., 2016). We demonstrated that
105 accession-specific differences in the ability to cope, counteract and/or recover from Cu- and Cd-
106 induced alterations to Cu homeostasis might be key to exhibit different metal sensitivity levels.
107 Overall, roots of Ws plants seemed to be better at counteracting the alterations to Cu homeostasis
108 by sequestering excess Cu or remobilizing intracellular Cu more effectively than roots of Col-0
109 plants exposed to excess Cu or Cd (Amaral dos Reis et al., submitted).

110 Although roots are the first plant organ coming into contact with metals in the soil and are
111 therefore directly affected, leaves also display metal-induced effects due to root-to-shoot metal
112 translocation and/or inter-organ signaling. In the current study, responses in the leaves of Col-0
113 and Ws plants exposed to metal excess are compared to complement our previous study on roots
114 and reveal accession-specific changes. More specifically, effects of excess Cu and Cd on rosette
115 growth, metal concentrations and transport, and their associated oxidative stress signatures were
116 determined in both accessions after exposure for 24 and 72 h.

117 **2. Material and methods**

118 **2.1. Plant material**

119 *Arabidopsis thaliana* (L.) Heynh plants, accessions Columbia (Col-0) and Wassilewskija
120 (Ws), were grown on sand in a hydroponic system (based on the method described by Smeets et
121 al. (2008)), at 22 °C/18 °C during 12 h/12 h light/dark periods respectively, with a light intensity
122 of 170 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the rosette level and 65 % relative humidity.

123 Nineteen-day-old plants of each accession were exposed to sublethal Cu or Cd concentrations
124 (Cuypers et al., 2011; Smeets et al., 2008), 2 μM (excess) CuSO_4 or 5 μM CdSO_4 via the roots, or
125 further grown under non-exposed (“control”) conditions (32 nM CuSO_4). These Cu and Cd
126 concentrations are environmentally realistic and comparable to those commonly found in pore
127 water of contaminated soils in North Limburg (Belgium). Leaf (*i.e.* whole rosette) samples were
128 harvested 24 and 72 h after the start of metal exposure. Biological replicates were sampled from –
129 depending on the required sample weight – one or more individual plants out of one pot at each
130 time point. To avoid within-pot correlation (Smeets et al., 2008), different biological replicates
131 were sampled out of at least two pots containing the same Cu or Cd concentration. Samples to
132 determine Cu and Cd concentrations were processed as described hereafter, while the remaining
133 samples were snap frozen in liquid nitrogen and stored at - 70 °C for the remaining analyses.

134 **2.2. Determination of metal concentrations**

135 Leaf samples were first rinsed in distilled water and then dried at 60 °C for at least one week.
136 Dried leaf samples were digested in HNO_3 (65 %) in a microwave oven (CEM MDS-2000
137 Microwave Digestor Oven, CEM Corporation, NC, USA), followed by a five-fold dilution in
138 ultrapure water. The Cu and Cd concentrations were determined by atomic absorption

139 spectrophotometry (Unicam Solaar M, Thermo Fisher Scientific, Inc., MA, USA) in the acid-
140 digested samples, employing a graphite furnace for Cd concentration assessment.

141 The ratio between metal concentrations in the leaves and in the roots of the same plants was
142 calculated to estimate root-to-shoot translocation factors of Cu and Cd in both accessions.

143 **2.3. Enzymatic activity determination**

144 Spectrophotometric (UV-1800 UV-VIS Spectrophotometer, Shimadzu Corporation, Kyoto,
145 Japan) methods were used to estimate the activities of superoxide dismutase (SOD), catalase
146 (CAT), glutathione reductase (GR), guaiacol peroxidase (GPOD), syringaldazine peroxidase
147 (SPOD), malic enzyme (ME), isocitrate dehydrogenase (ICDH) and glucose-6-phosphate
148 dehydrogenase (G6PDH) in purified protein extracts. Enzyme activities were calculated
149 according to the Lambert-Beer law and defined as the amount of CAT, GR, GPOD, SPOD, ME,
150 ICDH or G6PDH needed for the conversion of 1 μmol of substrate per min and cm^3 at room
151 temperature, or as the amount of SOD necessary to inhibit the reduction of cytochrome c by 50 %
152 per min and cm^3 at room temperature.

153 Leaf samples were first crushed in liquid nitrogen, using a mortar and pestle, and then
154 homogenized in a 0.1 M Tris-HCl (pH 7.8) solution containing 5 mM EDTA, 1 % (w/v)
155 polyvinylpyrrolidone (PVP) K30, 5 mM dithioerythritol (DTE) and 1 % (V/V) Nonidet P-40.
156 After agitation for 30 min, leaf homogenates were centrifuged at 50 000 x g and 4 °C for 30 min
157 to obtain a crude protein extract (supernatant). The proteins were then fractionated from the crude
158 extract in a two-step ammonium sulfate precipitation method (first by 40 % and second by 80 %
159 salt saturation), each step performed for 30 min at 4 °C and followed by centrifugation at 50 000
160 x g and 4 °C. Finally, the pelleted proteins were resuspended in a 25 mM Tris-HCl (pH 7.8)

161 buffer. Purified extracts were desalted using PD-10 Desalting Columns (GE Healthcare, Illinois,
162 USA), snap-frozen in liquid nitrogen and stored at - 70 °C until the enzyme measurements.

163 Superoxide dismutase (EC 1.15.1.1) activity was assessed at 550 nm in a reaction mixture of
164 50 mM KH₂PO₄ (pH 7.8) buffer, 0.1 mM EDTA, 10 μM cytochrome c, 50 μM xanthine and
165 7.2 mU xanthine oxidase (EC 1.17.3.2) (McCord and Fridovich, 1969). Catalase (EC 1.11.1.6)
166 activity was measured at 240 nm ($\epsilon_{\text{H}_2\text{O}_2} = 40 \text{ mM}^{-1} \text{ cm}^{-1}$) using 0.1 M KH₂PO₄ (pH 7.0) buffer
167 and 0.85 mM H₂O₂ (Bergmeyer et al., 1974). Guaiacol peroxidase (EC 1.11.1.7) activity was
168 determined at 436 nm ($\epsilon_{\text{tetraguaiacol}} = 25.5 \text{ mM}^{-1} \text{ cm}^{-1}$), in a reaction mixture containing a 0.1 M
169 KH₂PO₄ (pH 7.0) buffer, 0.8 mM H₂O₂ and 1.8 mM guaiacol (Bergmeyer et al., 1974).
170 Syringaldazine peroxidase (EC 1.11.1.7) activity was measured at 530 nm ($\epsilon_{\text{oxidized syringaldazine}} =$
171 $11.6 \text{ mM}^{-1} \text{ cm}^{-1}$), with the reaction mixture consisting of 0.1 M Tris-HCl (pH 7.5) buffer, 1 mM
172 H₂O₂, 56.6 μM syringaldazine, 0.13 M 1,4-dioxane and 0.14 M methanol (Imberty et al., 1984).
173 Activities of GR, ME, ICDH and G6PDH were determined at 340 nm ($\epsilon_{\text{NADPH}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).
174 For glutathione reductase (EC 1.8.1.7), the reaction mixture included 0.1 M Tris and 1 mM
175 EDTA (pH 8.0) buffer, 1.4 mM GSSG and 0.1 μM NADPH (Bergmeyer et al., 1974). Malic
176 enzyme (EC 1.1.1.39) activity was assessed using 15 mM Tris-HCl (pH 7.3) buffer, 36 mM
177 MnSO₄, 10 mM NADP⁺ and 0.1 M L-malate (Bergmeyer et al., 1974). For isocitrate
178 dehydrogenase (EC 1.1.1.42) the reaction mixture contained 0.1 M Tris (pH 7.5) buffer, 4.6 mM
179 DL-isocitrate, 52 mM NaCl and 5 mM NADP⁺ (Bergmeyer et al., 1974). Finally, glucose-6-
180 phosphate dehydrogenase (EC 1.1.1.49) activity was estimated using 50 mM Tris-HCl (pH 7.6)
181 buffer, 1 mM glucose-6-phosphate, 0.2 mM NADP⁺ and 6.7 mM MgCl₂ (Bergmeyer et al.,
182 1974).

183 **2.4. Determination of glutathione concentrations**

184 Oxidized (glutathione disulfide, GSSG) and reduced (GSH) forms of glutathione were
185 analyzed on a plate reader by enzymatic assays based on the protocol described by Queval and
186 Noctor (2007). Leaf samples were crushed in liquid nitrogen using a mortar and pestle, followed
187 by homogenization in 200 mM HCl (pH 4.5). After reduction of GSSG to GSH by GR, total GSH
188 and GSSG concentrations were determined by a kinetic enzymatic recycling assay based on the
189 GSH-dependent reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The absorbance at 412
190 nm was measured in a 200 mM NaH₂PO₄ and 10 mM EDTA (pH 7.5) buffer containing 0.6 mM
191 DTNB (in dimethyl sulfoxide), 0.5 mM NADPH and 1 U mL⁻¹ GR. The rate of absorbance
192 change over 5 min is proportional to the GSH concentration in the sample, which was calculated
193 using GSH and GSSG standard curves ranging from 0 to 1 or 0.4 nmol, respectively. To
194 determine the concentration of GSSG, the extracts (and GSSG standards) were first incubated in
195 1 % (V/V) 2-vinyl-pyridine (2-VP) at 20 °C for 30 min to derivatize GSH and then twice
196 centrifuged for 10 min at 16 100 x g and 4 °C to precipitate 2-VP.

197 **2.5. Gene expression analysis**

198 Frozen leaf samples were shredded twice in liquid nitrogen-cooled adapters for 1 min at 30/s
199 frequency (Retsch Mixer Mill MM400, Verder Scientific GmbH & Co. KG, Haan, Germany).
200 Next, RNA was extracted from the homogenized samples using the Ambion™ RNAqueous® Kit
201 (Life Technologies, Waltham, MA, USA) and eluted in RNase-free water pre-heated at 80 °C.
202 Concentration and quality of the RNA samples were verified using the NanoDrop ND-1000
203 spectrophotometer (Thermo Fisher Scientific, Inc., MA, USA). Prior to cDNA synthesis, the
204 RNA samples were cleaned of genomic DNA using the TURBO DNA-free™ Kit (Life

205 Technologies). Complementary DNA was synthesized from equal amounts (1.1 µg) of cleaned
206 RNA samples using the PrimeScript™ RT Reagent Kit (Perfect Real Time) (TAKARA BIO Inc.,
207 Shiga, Japan) and the thermal cycler Techne TC-5000 (Life Technologies). The cDNA samples
208 were diluted in 1/10 TE (Tris-EDTA) buffer and stored at - 20 °C.

209 Real-time quantitative PCR (qPCR) analysis was performed using the Applied Biosystems™
210 Fast SYBR® Master Mix (Thermo Fisher Scientific, Inc.) and 300 (or 600 nM) of gene-specific
211 forward and reverse primers (Supplementary Tables 1 and 2). The amplification reaction
212 involved 40 cycles of denaturation at 95 °C for 3 s followed by annealing/elongation at 60 °C for
213 30 s, after an initial denaturation at 95 °C for 20 s, and was performed in the Applied
214 Biosystems™ 7500 Fast Real Time PCR System (Life Technologies). Subsequently, a melting
215 curve was generated to verify amplification specificity. After analyzing five candidate reference
216 genes (Remans et al., 2008), *AT2G28390*, *AT4G05320*, and *AT5G15710* were selected using the
217 GrayNorm algorithm (Remans et al., 2014) to normalize the expression levels of the genes of
218 interest (Supplementary Tables 1 and 2). Expression levels were determined for several genes of
219 interest (Supplementary Table 2): five oxidative stress hallmark genes (Gadjev et al., 2006); two
220 genes encoding ROS-producing enzymes; five encoding antioxidative enzymes; two primary
221 microRNA transcripts; three encoding copper transporters; five encoding metallothioneins; three
222 encoding protein kinases; two encoding transcription factors; and one gene encoding a protein
223 involved in ethylene signaling.

224 Hierarchical clustering analysis was performed (GenEx Pro software, v6.1, MultiD Analyses
225 AB, Sweden) to recognize potential sample-related patterns within leaves of plants of both
226 accessions exposed to excess Cu and Cd. The analysis was based on raw gene expression values
227 (Cq values). The distance between conditions was defined by the “Average linkage” algorithm as

228 the average of distances between all pairs of individuals in all groups, while the distances
229 between the measures were calculated via the Euclidian Distance Measure. Heat maps were
230 constructed to compare expression levels between different genes and samples.

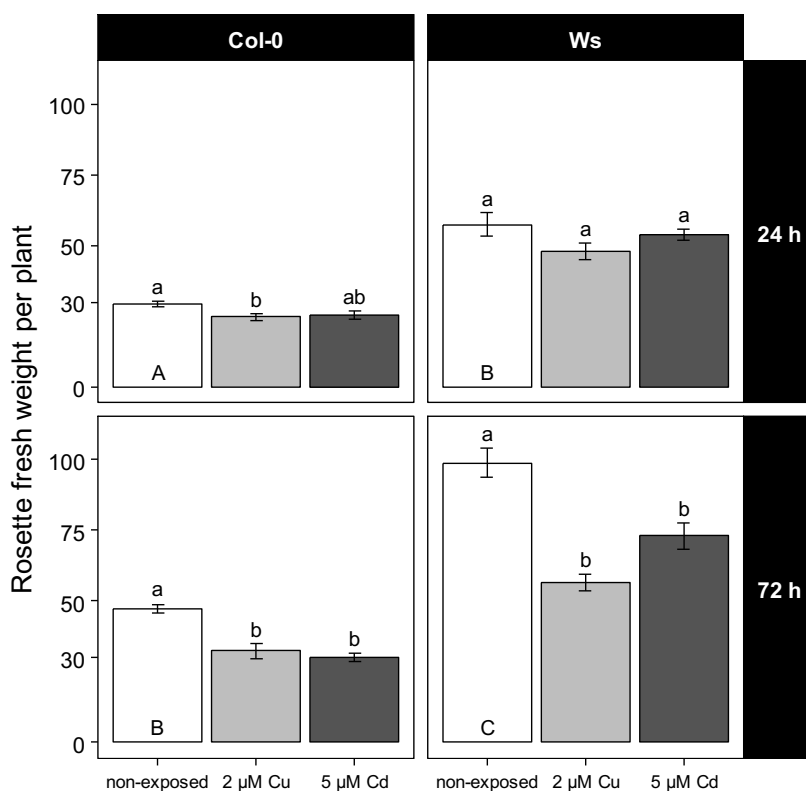
231 **2.6. Statistical analyses**

232 Statistical analysis of data obtained from rosette growth measurements, determination of metal
233 concentrations, enzyme analysis, glutathione concentration measurements, and gene expression
234 analysis was performed using R 3.3.1 (R Core Team, 2016) running on RStudio 1.0.143 (RStudio
235 Team, 2015). Data normality was tested using the Shapiro-Wilk test, while homoscedasticity was
236 verified via Bartlett's and Levene's tests. Normally distributed datasets were analyzed via one-
237 way ANOVA, followed by post-hoc analysis via the Tukey's HSD test when significant. Non-
238 normal datasets were analyzed via the Kruskal-Wallis test. To determine statistical significance
239 of gene expression data, datasets were first log-transformed.

240 **3. Results**

241 **3.1. Rosette growth**

242 Hydroponically grown three-weeks-old *A. thaliana* plants of Col-0 and Ws background were
243 exposed to excess Cu (2 μ M) or Cd (5 μ M). To determine responses after short-term and more
244 prolonged metal exposure, leaves were sampled 24 and 72 h after the onset of Cu or Cd exposure.



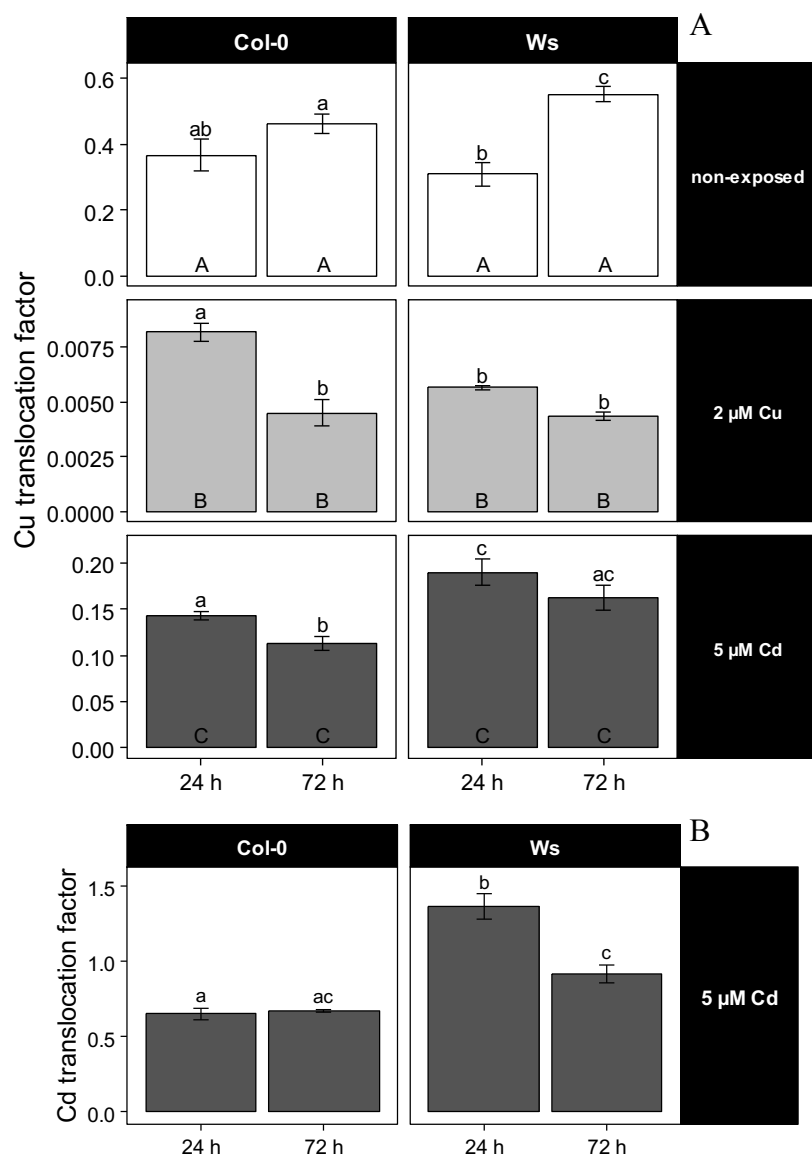
245
246 **Fig. 1.** Rosette fresh weight per plant (in mg) of three-weeks-old *A. thaliana* plants (accessions
247 Col-0 and Ws) exposed to 2 μ M CuSO₄, 5 μ M CdSO₄ or not exposed for 24 and 72 h. Values are
248 mean \pm S.E. of at least three biological replicates, each containing rosettes of 25 individual
249 plants. \square = non-exposed. \blacksquare = exposed to 2 μ M CuSO₄. \blacksquare = exposed to 5 μ M CdSO₄. Statistical
250 significance ($P < 0.05$) is indicated using lowercase (within accession and time point) or
251 uppercase letters (between non-exposed accessions at both time points).

252 *Arabidopsis* natural accessions Col-0 and Ws are morphologically different (Passardi et al.,
253 2007). Under non-exposed conditions, rosettes of Ws plants had a significantly higher fresh
254 weight as compared to those of Col-0 plants (Fig. 1). Moreover, this was not related to a different

255 number of leaves, but rather to a larger surface area of the leaves of Ws plants versus Col-0 plants
256 (data not shown). Excess Cu and Cd inhibited rosette growth in both accessions as indicated by a
257 lower fresh weight (Fig. 1). While exposure to excess Cu resulted in an inhibition of Col-0 rosette
258 growth already after 24 h, Ws rosettes were only significantly affected by Cu after 72 h. The
259 effects of Cd exposure were only significant in leaves of both Col-0 and Ws plants after 72 h
260 (Fig. 1).

261 **3.2. Metal translocation factors**

262 To evaluate the ability to translocate Cu and/or Cd, the translocation factors were estimated
263 (Fig. 2) from the concentrations of Cu and Cd in roots and leaves (Supplementary Table 3) of
264 non-exposed, Cu- and Cd-exposed plants. In non-exposed conditions, the translocation factor of
265 Cu significantly increased in Ws plants over time, with a similar trend in Col-0 plants (Fig. 2A).
266 Exposure to excess Cu severely impaired relative root-to-shoot Cu translocation as compared to
267 non-exposed conditions (Fig. 2A). Whereas the Cu translocation factor was significantly higher
268 in Col-0 than Ws plants after 24 h, it decreased to translocation factors similar to those of Ws
269 plants after 72 h. The root-to-shoot Cu translocation factor in Cu-exposed Ws plants decreased as
270 compared to non-exposed conditions but remained constant over time (Fig. 2A). Although the
271 root-to-shoot Cu translocation factor was reduced by Cd exposure in both accessions (Fig. 2A), it
272 was significantly higher in Ws than Col-0 plants at each time point (Fig. 2A). During exposure to
273 Cd, its translocation factor in Col-0 plants was constant over time (Fig. 2B). The root-to-shoot Cd
274 translocation factor was significantly higher in Ws than Col-0 plants after 24 h, but decreased to
275 values comparable to those in Col-0 plants after 72 h (Fig. 2B).



276

277 **Fig. 2.** Root-to-shoot translocation factors of Cu and Cd in three-weeks-old *A. thaliana* plants
 278 (accessions Col-0 and Ws) exposed to 2 μM CuSO₄, 5 μM CdSO₄ or not exposed for 24 and 72
 279 h. **(A)** Cu translocation factors in non-exposed, Cu- and Cd-exposed plants. **(B)** Cd translocation
 280 factors in Cd-exposed plants. Values are mean ± S.E. of at least three biological replicates. □ =
 281 non-exposed. ■ = exposed to 2 μM CuSO₄. ■ = exposed to 5 μM CdSO₄. Statistical significance
 282 ($P < 0.05$) is indicated using different lowercase (within exposure condition, between accessions
 283 and time points) or uppercase letters (across exposure conditions, within accession and time
 284 point).

285 3.3. Activities of antioxidative and NAD(P)H-producing enzymes

286 Activities of antioxidative enzymes (SOD, CAT, GR, GPOD and SPOD) and of NAD(P)H-
 287 producing enzymes (ICDH, ME and G6PDH) were determined in leaves of Col-0 and Ws plants
 288 (Table 1). Exposure to excess Cu only affected the enzymatic activities in leaves of Ws plants.
 289 Superoxide dismutase activity was significantly higher after exposure to Cu for 72 h, as were GR
 290 and ME activities at both time points. Exposure to Cd led to an increased ME activity in leaves of
 291 Col-0 and Ws plants at 24 and 72 h. The activities of GR, GPOD, SPOD, ICDH and G6PDH
 292 were only significantly higher in leaves of Col-0 plants after 72 h of Cd exposure (Table 1).

293 **Table 1.** Activities of antioxidative and redox-regulating enzymes (mU mg⁻¹ fresh weight) in
 294 leaves of three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws) exposed to 2 μM CuSO₄,
 295 5 μM CdSO₄ or not exposed for 24 and 72 h. Antioxidative enzymes: superoxide dismutase
 296 (SOD), catalase (CAT), glutathione reductase (GR), guaiacol peroxidase (GPOD) and
 297 syringaldazine peroxidase (SPOD). NAD(P)H-producing enzymes: isocitrate dehydrogenase
 298 (ICDH), malic enzyme (ME) and glucose-6-phosphate dehydrogenase (G6PDH). Values are
 299 mean ± S.E. of at least three biological replicates, each containing rosettes of at least two
 300 individual plants. Statistical significance (P < 0.05) is indicated using different lowercase letters
 301 (for differences within each accession and time point).

	Col-0			Ws			
	non-exposed	2 μM Cu	5 μM Cd	non-exposed	2 μM Cu	5 μM Cd	
SOD	24 h	367.76 ± 40.50 a	360.55 ± 18.92 a	317.46 ± 3.74 a	301.20 ± 33.15 a	361.16 ± 31.59 a	299.82 ± 7.10 a
	72 h	249.82 ± 70.80 a	251.6 ± 15.27 a	254.87 ± 18.20 a	188.26 ± 43.72 a	362.51 ± 14.07 b	257.89 ± 28.82 ab
CAT	24 h	1.24 ± 0.40 a	0.71 ± 0.20 a	3.71 ± 1.37 a	1.03 ± 0.57 a	13.80 ± 7.55 a	2.51 ± 0.99 a
	72 h	1.98 ± 1.13 a	3.59 ± 2.01 a	6.32 ± 1.66 a	2.72 ± 0.98 a	1.34 ± 0.39 a	2.64 ± 1.10 a
GR	24 h	872.14 ± 47.96 a	1073.43 ± 97.31 a	1133.84 ± 0.05 a	851.58 ± 122.55 a	1283.30 ± 70.17 b	1055.06 ± 67.42 ab
	72 h	828.67 ± 39.95 a	850.93 ± 36.70 a	1075.80 ± 47.91 b	634.20 ± 176.64 a	1168.64 ± 46.39 b	953.59 ± 31.51 ab
GPOD	24 h	23.64 ± 4.06 a	52 ± 14.12 a	42.06 ± 8.66 a	17.88 ± 7.06 a	47.98 ± 10.97 a	36.66 ± 7.82 a
	72 h	15.82 ± 3.94 a	19.62 ± 2.31 a	243.45 ± 16.19 b	6.93 ± 3.26 a	37.34 ± 9.04 a	36.75 ± 8.68 a
SPOD	24 h	57.08 ± 23.18 a	115.99 ± 49.15 a	219.87 ± 63.35 a	102.93 ± 43.91 a	172.16 ± 40.75 a	207.41 ± 45.11 a
	72 h	89.49 ± 18.23 a	102.54 ± 11.86 a	1421.37 ± 127.49 b	110.81 ± 22.4 a	258.37 ± 56.62 a	189.89 ± 56.43 a
ICDH	24 h	559.68 ± 25.79 a	588.77 ± 55 a	647.77 ± 24.68 a	618.51 ± 218.03 a	549.57 ± 17.21 a	492.95 ± 25.93 a
	72 h	450.08 ± 38.11 a	483.27 ± 20.55 a	615.23 ± 19.18 b	330.77 ± 59.84 a	446.75 ± 20.71 a	402.64 ± 8.68 a
ME	24 h	242.72 ± 6.70 a	285.70 ± 76.98 a	501.06 ± 49.35 b	154.88 ± 23.55 a	293.80 ± 8.03 b	230.86 ± 9.90 c
	72 h	220.22 ± 10.33 a	357.39 ± 18.44 a	771.33 ± 132.53 b	169.89 ± 22.83 a	349.42 ± 28.93 b	397.00 ± 1.77 b
G6PDH	24 h	105.96 ± 7.61 a	138.12 ± 13.80 a	136.36 ± 10.97 a	60.76 ± 16.62 a	140.10 ± 46.75 a	115.95 ± 11.28 a
	72 h	95.06 ± 6.34 a	109.37 ± 0.87 a	168.55 ± 18.84 b	73.75 ± 27.27 a	150.68 ± 14.85 a	118.88 ± 3.88 a

302 **3.4. Glutathione concentrations**

303 Concentrations of reduced (GSH) and oxidized (glutathione disulfide, GSSG) glutathione
 304 were determined in leaves of both accessions (Table 2). Total glutathione levels were higher in
 305 the leaves of Col-0 plants after exposure to excess Cu for 72 h, which coincided with an
 306 increased GSSG concentration. In case of Col-0 plants exposed to Cd for 72 h, higher versus
 307 lower concentrations were observed for GSH and GSSG respectively, resulting in a lower
 308 GSSG/GSH ratio (Table 2). No significant changes in total glutathione concentrations were
 309 observed in leaves of Ws plants. However, similar to Col-0 plants, a higher GSSG concentration
 310 was observed in leaves of Cu-exposed Ws plants after 72 h, resulting in a higher GSSG/GSH
 311 ratio. Exposure to Cd significantly lowered the GSSG concentration in leaves of Ws plants (Table
 312 2).

313 **Table 2.** Concentrations of total (GSH + GSSG), reduced (GSH), oxidized (GSSG) glutathione
 314 (nmoles g⁻¹ fresh weight) and GSSG/GSH ratio in leaves of three-weeks-old *A. thaliana* plants
 315 (accessions Col-0 and Ws), exposed to 2 μM CuSO₄, 5 μM CdSO₄ or not exposed for 24 and 72
 316 h. Values are mean ± S.E. of at least three biological replicates, each containing rosettes of at
 317 least one individual plant. Statistical significance (P < 0.05) is indicated using different lowercase
 318 letters (for differences within each accession and time point).

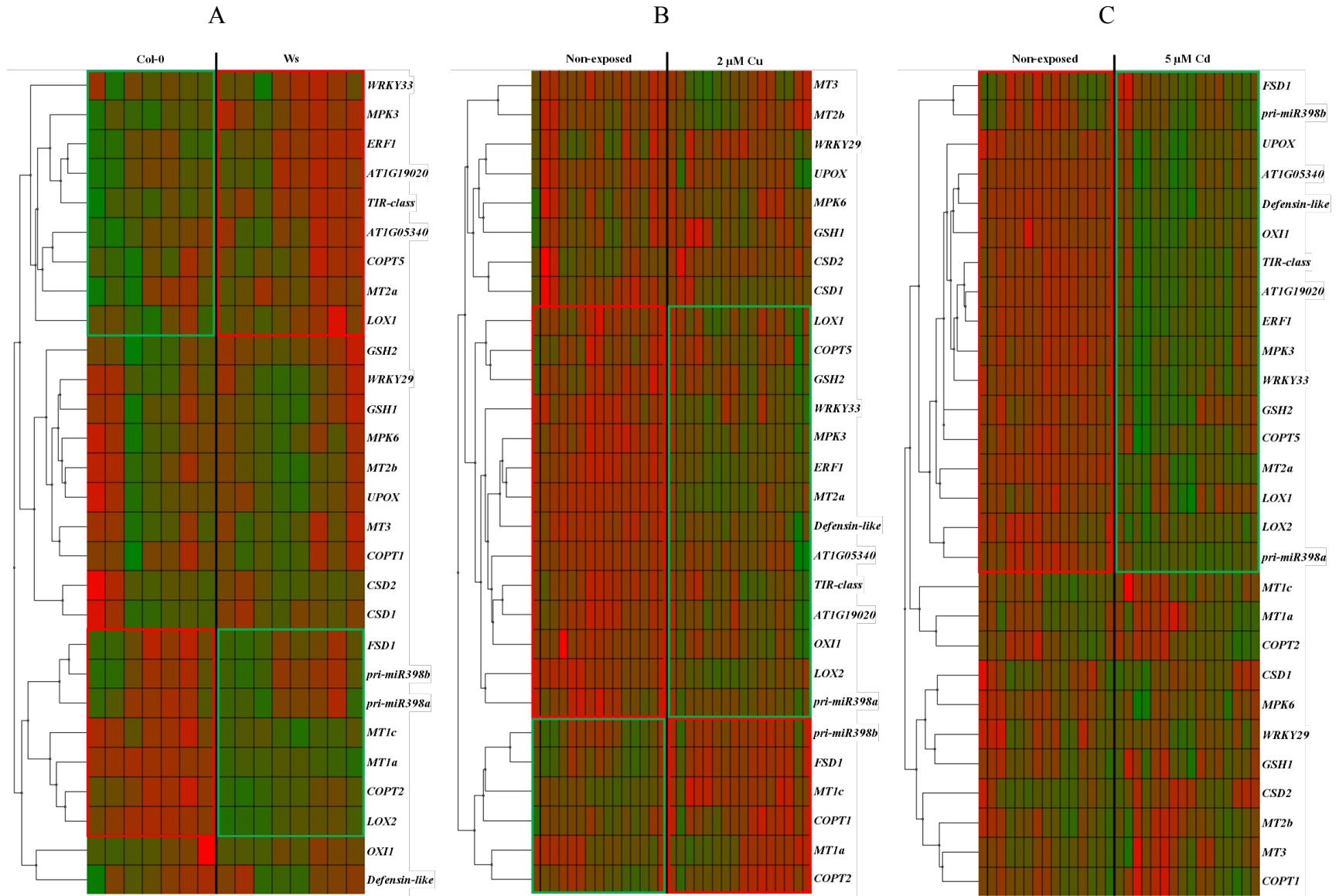
	Col-0			Ws			
	non-exposed	2 μM Cu	5 μM Cd	non-exposed	2 μM Cu	5 μM Cd	
Total (GSH+GSSG)	24 h	266.27 ± 55.68 a	266.5 ± 43.31 a	216.64 ± 44.62 a	212.71 ± 37.42 a	272.60 ± 87.22 a	208.77 ± 37.20 a
	72 h	229.71 ± 10.34 a	361.00 ± 37.53 b	419.32 ± 13.45 b	290.43 ± 35.95 a	301.74 ± 24.88 a	335.00 ± 66.45 a
GSH	24 h	250.93 ± 51.43 a	256.67 ± 39.82 a	213.58 ± 44.77 a	205.20 ± 37.58 a	261.07 ± 85.92 a	205.66 ± 37.37 a
	72 h	212.67 ± 11.15 a	328.90 ± 37.55 a	416.37 ± 13.03 b	280.44 ± 35.78 a	246.21 ± 5.74 a	330.05 ± 66.15 a
GSSG	24 h	15.34 ± 4.77 a	9.83 ± 3.91 a	3.06 ± 0.42 a	7.51 ± 0.86 ab	11.53 ± 2.16 a	3.11 ± 0.45 b
	72 h	15.84 ± 1.74 a	31.68 ± 0.58 b	6.25 ± 0.63 c	9.99 ± 0.83 a	55.54 ± 20.91 b	4.95 ± 0.43 b
GSSG/GSH	24 h	0.059 ± 0.011 a	0.035 ± 0.009 ab	0.011 ± 0.000 b	0.040 ± 0.011 a	0.068 ± 0.028 a	0.017 ± 0.005 a
	72 h	0.062 ± 0.004 a	0.075 ± 0.010 a	0.018 ± 0.002 b	0.037 ± 0.005 a	0.223 ± 0.080 b	0.016 ± 0.002 a

319

320 3.5. Gene expression

321 Expression levels of several genes involved in Cu transport and chelation (MTs), pro- and
322 antioxidative responses, secondary metabolism, ethylene/MAPK signaling pathways and genes
323 encoding transcription factors were determined in leaves of Col-0 and Ws plants either not
324 exposed (Table 3) or exposed to excess Cu (Table 4) or Cd (Table 5).

325 Representation of the gene expression data of leaves from plants grown under non-exposed
326 conditions using a heat map revealed an accession-related clustering, with Ws samples mostly
327 clustering separated from Col-0 samples (Fig. 3A; for more detail see Supplementary Fig. 1). In
328 addition, hierarchical clustering revealed two gene clusters responding differently in the leaves of
329 both accessions. One cluster included Cu homeostasis-related genes such as the iron superoxide
330 dismutase gene *FSD1*, primary microRNAs *pri-miR398a* and *pri-miR398b*, MT genes *MT1a* and
331 *MT1c* and the Cu transporter gene *COPT2*, and the lipoxygenase gene *LOX2* with higher
332 expression levels in leaves of Ws (green-shaded rectangles) than Col-0 plants (red-shaded
333 rectangles). Opposite expression patterns were observed within the other gene cluster grouping
334 three oxidative stress hallmark genes (Gadjev et al., 2006), MAPK/ethylene signaling-related
335 genes (mitogen-activated kinase gene *MPK3*, WRKY DNA-binding protein gene *WRKY33* and
336 ethylene response factor gene *ERF1*), the pro-oxidative gene *LOX1*, as well as *COPT5* and *MT2a*
337 (Fig. 3A). Transcript level analysis of both gene clusters confirmed accession-specific expression
338 levels in leaves of non-exposed Col-0 and Ws plants (Table 3).



340 **Fig. 3.** Heat map representations of the gene expression data obtained in leaves of three-weeks-
341 old *A. thaliana* plants (accessions Col-0 and Ws). (A) Heat map of data collected from plants
342 grown in non-exposed conditions for 24 and 72 h. (B) Heat map of data collected from plants
343 exposed to 2 μ M CuSO₄ or not exposed (C) Heat map of data collected from plants exposed to 5
344 μ M CdSO₄ or not exposed. Hierarchical clustering of genes is shown at the top (gene names at
345 the bottom). Green-shaded rectangles indicate increased, while red-shaded rectangles indicate
346 decreased gene expression. Abbreviations: *UPOX*: upregulated by oxidative stress; *Defensin-*
347 *like*: protein member of the defensin-like (*DEFL*) family; *AT1G19020*: unknown protein;
348 *AT1G05340*: unknown protein; *TIR-class*: Toll-Interleukin-Resistance (*TIR*) domain family
349 protein; *LOX1*: lipoxygenase 1; *GSH1*: glutamate-cysteine ligase; *GSH2*: glutathione synthetase
350 2; *CSD*: Cu/Zn superoxide dismutase; *FSD1*: Fe superoxide dismutase 1; *pri-miR398a*: primary
351 microRNA 398a; *pri-miR398b*: primary microRNA 398b; *COPT*: copper transporter; *MT*:
352 metallothionein; *OXII*: Oxidative signal inducible 1; *MPK*: mitogen-activated protein kinase;
353 *WRKY*: *WRKY* DNA-binding protein; *ERF1*: ethylene response factor 1.

354 Comparing non-exposed to Cu- or Cd-exposed samples revealed a separate clustering between
355 the metal-exposed and non-exposed groups (Figs. 3B and 3C; for more detail see Supplementary
356 Figs. 2 and 3). After including only non-exposed and Cu-exposed samples in the heat map, two
357 groups of genes emerged (Fig. 3B): (1) as indicated by the red-shaded rectangles, genes that were
358 less expressed after Cu exposure, including those involved in Cu homeostasis, such as *FSD1*, *pri-*
359 *miR398b*, *COPT1*, *COPT2* and *MT1c* (Cluster Cu-I); (2) as indicated by the green-shaded
360 rectangles, genes that were more expressed under excess Cu conditions, including those involved
361 in MAPK/ethylene signaling, such as *OXII*, *MPK3*, *WRKY33* and *ERF1*, four oxidative stress
362 hallmark genes, *pri-miR398a*, *LOX1* and *LOX2*, *GSH2* and *MT2a* (Cluster Cu-II). Moreover,
363 gene expression data revealed some accession-specific changes in the expression of these genes
364 upon exposure to excess Cu (Table 4). Concerning the genes in cluster Cu-I, transcript levels of
365 *FSD1*, *pri-miR398b* and *COPT2* were significantly lower only in leaves of Cu-exposed Ws plants
366 after 72 h (Table 4). Expression of *MT1c* was significantly lower in leaves of Ws plants exposed
367 to excess Cu at both time points (Table 4). Regarding the genes in cluster Cu-II, transcript levels
368 of *GSH2* and *OXII* were significantly higher only in leaves of Cu-exposed Col-0 plants (at 72
369 and 24 h respectively) (Table 4). Moreover, while *pri-miR398a* transcript levels were higher after

370 24 h of Cu exposure in leaves of both accessions, its upregulation was significantly higher in Col-
371 0 as compared to Ws plants (Table 4). Transcript levels of *MPK3* and *WRKY33* were higher in
372 leaves of Cu-exposed Ws plants at both time points. In Col-0 plants, *MPK3* was only upregulated
373 after 24 h, whereas *WRKY33* was significantly upregulated after 72 h of exposure to excess Cu
374 (Table 4). The ethylene signaling-related gene *ERF1* was significantly upregulated after Cu
375 exposure and its transcript levels were significantly higher in leaves of Ws as compared to Col-0
376 plants (Table 4).

377 Leaves of Cd-exposed Col-0 and Ws plants generally clustered away from leaves of plants
378 grown under non-exposed conditions (Fig. 3C). Several genes grouped together due to their
379 higher expression upon Cd exposure (green-shaded rectangles). This cluster included oxidative
380 stress-related genes such as all five oxidative stress hallmark genes, *GSH2*, *LOX1* and *LOX2*, the
381 MAPK/ethylene signaling-related genes (*OXII*, *MPK3*, *WRKY33* and *ERF1*), *COPT5*, *FSD1*,
382 *MT2a* and *pri-miR398a/b* (Fig. 3C). Moreover, gene expression analysis revealed some
383 accession-specific differences in their expression upon Cd exposure (Table 5). Transcript levels
384 of oxidative stress hallmark genes (Gadjev et al., 2006) and ethylene signaling-related gene *ERF1*
385 were generally more upregulated in leaves of Col-0 than Ws plants after Cd exposure (Table 5).
386 Transcript levels of *LOX1* were significantly higher in leaves of Col-0 plants after 72 h exposure
387 to Cd. While *LOX2* and *pri-miR398a* were upregulated in the leaves of both accessions after 24 h,
388 their transcript levels were significantly higher in Col-0 than in Ws plants and remained
389 upregulated after 72 h in the former accession (Table 5). Cadmium-induced upregulation of the
390 *GSH2* gene occurred in leaves of Col-0 plants only. Exposure to Cd led to significantly increased
391 expression levels of *MT2a* in leaves of both accessions after 72 h. Although *OXII* expression
392 increased in leaves of both accessions exposed to Cd, its transcript levels were significantly
393 higher in leaves of Col-0 than Ws plants after 24 h (Table 5).

394 Although not included in one of the above-mentioned gene clusters (Fig. 3), *CSD1*, *MT2b*,
395 *MT3* and *WRKY29* were differentially expressed in leaves of Col-0 and Ws plants exposed to
396 excess Cu (Table 4) or Cd (Table 5). On the one hand, exposure to Cu increased *CSD1*
397 expression in leaves of Ws plants after 24 h (Table 4). Furthermore, *MT2b* and *MT3* were
398 upregulated in leaves of Ws plants after 72 h (Table 4). On the other hand, exposure to Cd alone
399 caused upregulation of *WRKY29* in leaves of Col-0 plants after 72 h (Table 5).

400 **Table 3.** Transcript levels of genes within clusters identified in leaves of three-weeks-old *A.*
401 *thaliana* plants (accessions Col-0 and Ws) grown under non-exposed conditions. Values are mean
402 normalized expression of Col-0 samples at 24 h \pm S.E. (abundance, within gene family) or
403 relative to Col-0 samples at 24 h (set at 1.00) \pm S.E (fold-change) of at least three biological
404 replicates, each containing rosettes of at least one individual plant. Resolution values are mean
405 inverse normalization factors relative to the non-exposed at each time point, indicating the
406 stability of the selected reference genes. Statistical significance ($P < 0.05$) is indicated by
407 asterisks and printed in bold (for differences within each time point, between accessions).
408 Abbreviations: see Fig. 3.

Col-0		Non-exposed	Ws	
24 h	72 h		24 h	72 h
1.00 \pm 0.18	1.48 \pm 0.17	Resolution	1.08 \pm 0.10	1.40 \pm 0.19
Genes encoding oxidative stress hallmark proteins				
1.00 \pm 0.13	3.77 \pm 1.28	<i>ATIG19020</i>	0.51 \pm 0.04 *	1.86 \pm 0.22
1.00 \pm 0.13	1.73 \pm 0.38	<i>ATIG05340</i>	0.86 \pm 0.06	1.29 \pm 0.09
1.00 \pm 0.38	3.88 \pm 2.41	<i>TIR-class</i>	0.18 \pm 0.03 *	0.66 \pm 0.21
Genes encoding ROS-producing enzymes				
1.00 \pm 0.22	1.22 \pm 0.15	<i>LOX1</i>	0.70 \pm 0.09	1.14 \pm 0.11
1.00 \pm 0.17	2.14 \pm 0.43	<i>LOX2</i>	2.47 \pm 0.20 *	4.85 \pm 0.38 *
Gene encoding antioxidative enzyme				
1.00 \pm 0.48	15.09 \pm 8.20	<i>FSD1</i>	2.24 \pm 0.82	15.21 \pm 3.04
Primary microRNA transcripts				
1.00 \pm 0.22	46.45 \pm 28.43	<i>pri-miR398a</i>	3.97 \pm 0.17 *	94.66 \pm 25.62
1.00 \pm 0.22	15.31 \pm 7.69	<i>pri-miR398b</i>	0.92 \pm 0.21	11.75 \pm 2.25
Genes encoding copper transporters				
1.00 \pm 0.08	2.58 \pm 0.98	<i>COPT2</i>	3.16 \pm 0.37 *	7.09 \pm 0.48 *
1.00 \pm 0.10	1.28 \pm 0.27	<i>COPT5</i>	0.74 \pm 0.06	1.09 \pm 0.05
Genes encoding metallothioneins				
1.00 \pm 0.21	1.81 \pm 0.13	<i>MT1a</i>	5.46 \pm 0.82 *	9.94 \pm 2.87 *
1.00 \pm 0.02	1.38 \pm 0.01	<i>MT1c</i>	2.13 \pm 0.24 *	2.36 \pm 0.30 *
1.00 \pm 0.08	1.65 \pm 0.32	<i>MT2a</i>	1.01 \pm 0.07	1.37 \pm 0.19
Gene encoding protein kinase				
1.00 \pm 0.22	1.41 \pm 0.35	<i>MPK3</i>	0.53 \pm 0.02 *	0.75 \pm 0.10
Gene encoding transcription factor				
1.00 \pm 0.25	1.94 \pm 0.62	<i>WRKY33</i>	0.55 \pm 0.04	1.48 \pm 0.21
Gene encoding protein involved in ethylene signaling				
1.00 \pm 0.13	4.38 \pm 1.03	<i>ERF1</i>	0.49 \pm 0.05 *	1.68 \pm 0.19 *

409

410 **Table 4.** Relative gene expression levels in leaves of three-weeks-old *A. thaliana* plants
 411 (accessions Col-0 and Ws), exposed to 2 μ M CuSO₄ for 24 and 72 h. Values are mean
 412 normalized expression relative to the non-exposed accession at each time point (set at 1.00) \pm
 413 S.E. of at least three biological replicates, each containing rosettes of at least one individual plant.
 414 Resolution values are mean inverse normalization factors relative to the non-exposed accession at
 415 each time point, indicating the stability of the selected reference genes. Statistically significant (P
 416 < 0.05) metal-induced changes in expression relative to the non-exposed accession at each time
 417 point are indicated by color (■ = upregulation; ■ = downregulation). Statistically significant (P $<$
 418 0.05) differences between accessions and within metal exposure are indicated by asterisks and
 419 printed in black and bold. Abbreviations: see Fig. 3.

Col-0		2 μ M Cu	Ws	
24 h	72 h		24 h	72 h
1.49 \pm 0.35	0.95 \pm 0.21	Resolution	1.4 \pm 0.39	1.11 \pm 0.29
Genes encoding oxidative stress hallmark proteins				
1.38 \pm 0.09	3.98 \pm 1.49	<i>UPOX</i>	1.06 \pm 0.00	2.02 \pm 0.64
5.31 \pm 0.53	7.86 \pm 5.90	<i>Defensin-like</i>	3.99 \pm 0.90	4.03 \pm 0.52
6.07 \pm 1.14	3.90 \pm 1.77	<i>ATIG19020</i>	5.29 \pm 1.77	3.40 \pm 1.10
2.94 \pm 0.48	8.97 \pm 4.54	<i>ATIG05340</i>	3.95 \pm 0.88	1.98 \pm 0.24
16.6 \pm 3.71	4.35 \pm 2.04	<i>TIR-class</i>	16.03 \pm 6.58	2.92 \pm 1.27
Genes encoding ROS-producing enzymes				
1.42 \pm 0.06	1.29 \pm 0.23	<i>LOX1</i>	2.22 \pm 0.24	1.41 \pm 0.30
9.89 \pm 0.45	2.70 \pm 0.60	<i>LOX2</i>	5.33 \pm 0.65	2.44 \pm 0.55
Genes encoding antioxidative enzymes				
1.32 \pm 0.10	1.06 \pm 0.05	<i>GSH1</i>	1.24 \pm 0.05	0.80 \pm 0.05 *
1.39 \pm 0.12	1.65 \pm 0.11	<i>GSH2</i>	1.37 \pm 0.06	1.62 \pm 0.11
1.73 \pm 0.18	2.28 \pm 0.35	<i>CSD1</i>	2.34 \pm 0.21	1.81 \pm 0.60
0.96 \pm 0.19	1.37 \pm 0.05	<i>CSD2</i>	0.88 \pm 0.09	1.04 \pm 0.03
0.36 \pm 0.07	0.07 \pm 0.06	<i>FSD1</i>	0.21 \pm 0.07	0.17 \pm 0.15
Primary microRNA transcripts				
85.67 \pm 23.78	5.40 \pm 3.50	<i>pri-miR398a</i>	12.1 \pm 2.84 *	1.82 \pm 1.15
0.68 \pm 0.13	0.25 \pm 0.21	<i>pri-miR398b</i>	1.48 \pm 0.50	0.08 \pm 0.02
Genes encoding copper transporters				
0.90 \pm 0.06	0.73 \pm 0.05	<i>COPT1</i>	1.15 \pm 0.08	1.13 \pm 0.07
0.90 \pm 0.01	0.32 \pm 0.03	<i>COPT2</i>	1.04 \pm 0.11	0.66 \pm 0.02
1.38 \pm 0.13	1.11 \pm 0.19	<i>COPT5</i>	1.77 \pm 0.08	1.08 \pm 0.11
Genes encoding metallothioneins				
1.91 \pm 0.27	0.93 \pm 0.26	<i>MT1a</i>	1.55 \pm 0.03	1.67 \pm 0.46
0.70 \pm 0.14	0.45 \pm 0.15	<i>MT1c</i>	0.25 \pm 0.03	0.09 \pm 0.01
3.56 \pm 0.43	2.19 \pm 0.37	<i>MT2a</i>	4.14 \pm 0.45	3.7 \pm 0.36
1.22 \pm 0.08	1.17 \pm 0.29	<i>MT2b</i>	1.76 \pm 0.07 *	2.00 \pm 0.25
1.23 \pm 0.06	1.41 \pm 0.41	<i>MT3</i>	1.93 \pm 0.12 *	2.79 \pm 0.05

Genes encoding protein kinases				
10.65 ± 3.20	12.83 ± 6.78	<i>OXII</i>	2.22 ± 0.42	3.12 ± 1.54
2.77 ± 0.23	1.68 ± 0.18	<i>MPK3</i>	3.92 ± 0.58	3.37 ± 0.46
1.07 ± 0.04	1.24 ± 0.07	<i>MPK6</i>	1.26 ± 0.04	1.18 ± 0.13
Genes encoding transcription factors				
0.60 ± 0.14	2.12 ± 0.25	<i>WRKY29</i>	0.64 ± 0.13	1.28 ± 0.25
4.14 ± 1.21	4.32 ± 1.08	<i>WRKY33</i>	5.48 ± 1.62	3.10 ± 0.54
Gene encoding protein involved in ethylene signaling				
24.71 ± 2.85	4.76 ± 1.20	<i>ERF1</i>	86.51 ± 20.55 *	18.85 ± 5.43 *

420

421 **Table 5.** Relative gene expression levels in leaves of three-weeks-old *A. thaliana* plants
 422 (accessions Col-0 and Ws), exposed to 5 μM CdSO₄ for 24 and 72 h. Values are mean
 423 normalized expression relative to the non-exposed accession at each time point (set at 1.00) \pm
 424 S.E. of at least three biological replicates, each containing rosettes of at least one individual plant.
 425 Resolution values are mean inverse normalization factors relative to the non-exposed accession at
 426 each time point, indicating the stability of the selected reference genes. Statistically significant (P
 427 < 0.05) metal-induced changes in expression relative to the non-exposed accession at each time
 428 point are indicated by color (■ = upregulation; ■ = downregulation). Statistically significant (P
 429 < 0.05) differences between accessions and within metal exposure are indicated by asterisks and
 430 printed in black and bold. Abbreviations: see Fig. 3.

Col-0		5 μM Cd	Ws	
24 h	72 h		24 h	72 h
1.61 \pm 0.22	0.74 \pm 0.15		1.07 \pm 0.16	1.01 \pm 0.16
Genes encoding oxidative stress hallmark proteins				
5.93 \pm 1.16	6.27 \pm 2.19	<i>UPOX</i>	2.21 \pm 0.19	2.21 \pm 0.32
27.80 \pm 4.70	46.09 \pm 17.38	<i>Defensin-like</i>	6.81 \pm 0.60 *	8.93 \pm 2.33
129.71 \pm 43.01	9.28 \pm 4.87	<i>ATIG19020</i>	56.87 \pm 15.44	3.58 \pm 0.06
77.41 \pm 36.40	30.25 \pm 15.94	<i>ATIG05340</i>	7.82 \pm 2.30	4.14 \pm 1.55
231.62 \pm 80.13	9.90 \pm 4.53	<i>TIR-class</i>	422.48 \pm 106.43	8.84 \pm 1.00
Genes encoding ROS-producing enzymes				
1.99 \pm 0.37	1.90 \pm 0.18	<i>LOX1</i>	1.27 \pm 0.04	1.11 \pm 0.11
9.42 \pm 2.21	2.30 \pm 0.50	<i>LOX2</i>	2.24 \pm 0.08 *	1.95 \pm 0.51
Genes encoding antioxidative enzymes				
1.19 \pm 0.19	1.27 \pm 0.07	<i>GSH1</i>	0.92 \pm 0.05	0.94 \pm 0.09 *
3.31 \pm 0.35	2.05 \pm 0.27	<i>GSH2</i>	1.25 \pm 0.07	1.46 \pm 0.10
1.40 \pm 0.23	0.85 \pm 0.02	<i>CSD1</i>	1.25 \pm 0.14	0.67 \pm 0.06
0.61 \pm 0.17	0.22 \pm 0.01	<i>CSD2</i>	0.80 \pm 0.08	0.45 \pm 0.22
4.83 \pm 1.14	0.85 \pm 0.15	<i>FSD1</i>	1.92 \pm 0.60	0.55 \pm 0.28
Primary microRNA transcripts				
2330.58 \pm 267.36	13.67 \pm 0.86	<i>pri-miR398a</i>	831.04 \pm 180.57 *	5.22 \pm 2.00
7.51 \pm 0.39	1.72 \pm 0.15	<i>pri-miR398b</i>	5.00 \pm 0.48	2.05 \pm 0.10
Genes encoding copper transporters				
0.88 \pm 0.09	0.88 \pm 0.02	<i>COPT1</i>	1.12 \pm 0.08	1.16 \pm 0.10
1.41 \pm 0.19	1.15 \pm 0.27	<i>COPT2</i>	1.47 \pm 0.05	1.38 \pm 0.30
5.40 \pm 1.06	1.28 \pm 0.10	<i>COPT5</i>	2.76 \pm 0.17	1.24 \pm 0.16
Genes encoding metallothioneins				
1.07 \pm 0.27	0.45 \pm 0.16	<i>MT1a</i>	0.93 \pm 0.11	1.22 \pm 0.08
1.03 \pm 0.06	1.70 \pm 0.25	<i>MT1c</i>	0.65 \pm 0.09	0.61 \pm 0.22
3.30 \pm 0.44	2.22 \pm 0.22	<i>MT2a</i>	2.21 \pm 0.06	2.63 \pm 0.17
0.93 \pm 0.09	0.88 \pm 0.08	<i>MT2b</i>	0.94 \pm 0.05	1.32 \pm 0.21
0.74 \pm 0.12	0.88 \pm 0.07	<i>MT3</i>	0.94 \pm 0.14	1.30 \pm 0.34

Genes encoding protein kinases				
272.11 ± 75.02	62.59 ± 36.07	<i>OXII</i>	38.74 ± 1.32 *	5.94 ± 1.96
5.83 ± 1.49	2.24 ± 0.34	<i>MPK3</i>	5.35 ± 0.61	2.34 ± 0.38
2.28 ± 0.51	1.40 ± 0.06	<i>MPK6</i>	1.48 ± 0.12	1.11 ± 0.09
Genes encoding transcription factors				
1.02 ± 0.07	2.80 ± 0.33	<i>WRKY29</i>	0.93 ± 0.03	1.53 ± 0.32
41.42 ± 13.28	7.69 ± 3.75	<i>WRKY33</i>	18.64 ± 5.94	2.82 ± 0.29
Gene encoding protein involved in ethylene signaling				
759.48 ± 165.90	13.11 ± 4.95	<i>ERF1</i>	289.41 ± 46.10	12.64 ± 2.50

431

432 4. Discussion

433 *Arabidopsis thaliana* is a well-established model plant with several resources and tools
434 available for molecular and genetic studies. Natural phenotypic variation, manifested by different
435 natural accessions, is one such important resource. Therefore, morphological and physiological
436 differences amongst the most popular *Arabidopsis* accessions have been well described and, to a
437 more limited extent, their genetic variability is studied (Alonso-Blanco et al., 2016; Passardi et
438 al., 2007). In this study on accession-specific responses, morphological differences between both
439 accessions were evident from leaf growth data of non-exposed plants (Fig. 1). In addition to the
440 biometrical parameters, genetic differences between the two accessions were highlighted in a heat
441 map presenting gene expression data of non-exposed plants (Fig. 3A). Hierarchical clustering
442 revealed two clusters of genes with differential expression patterns in leaves of Col-0 and Ws
443 plants. The higher expressed genes in Col-0 as compared to Ws plants were hallmark genes for
444 oxidative stress or associated to the MAPK and ethylene signaling pathways, while the lower
445 expressed genes were related to Cu homeostasis (Fig. 3A and Table 3). These results suggest that
446 Col-0 and Ws plants employ different life strategies. While Ws plants appear to constitutively
447 invest in nutrient homeostasis, Col-0 plants invest more in detoxification responses, related to
448 oxidative stress signaling and antioxidative defense mechanisms. In particular, the constitutively
449 higher expressed Cu homeostasis-related genes in leaves of Ws plants (Fig. 3A) encode for
450 proteins that are either involved in the mobilization or sequestration of Cu, such as *COPT2*,
451 *MT1a* and *MT1c* (Table 3; Guo et al., 2008; Sancenón et al., 2003), or are part of the trademark
452 strategy to redirect Cu, from dispensable to essential cuproproteins under Cu deficiency
453 conditions, such as *FSD1*, *pri-miR398a* and *pri-miR398b* (Gielen et al., 2016; Yamasaki et al.,
454 2007, 2008, 2009). As nutrient homeostasis is important in metal sensitivity, this supports our

455 previous results suggesting that Ws plants are less sensitive to excess Cu and Cd than Col-0
456 plants due their ability to better counteract alterations in Cu homeostasis (Amaral dos Reis et al.,
457 submitted), including a perceived Cd-induced Cu deficiency (Gielen et al., 2016).

458 Exposure for 72 h, but not for 24 h, to Cd significantly inhibited rosette growth in both
459 accessions. A similar response was observed for Ws plants after exposure to excess Cu (Fig. 1).
460 This delayed effect is not surprising since leaves are not in direct contact with the metals in the
461 growth medium and, as such, metal-induced responses depend on root-to-shoot translocation of
462 the metals and/or inter-organ signaling. Exposure to excess Cu, however, significantly inhibited
463 rosette growth of Col-0 plants already after 24 h (Fig. 1), coinciding with a significantly higher
464 Cu concentration (Supplementary Table 3) and higher root-to-shoot Cu translocation factor at this
465 time point (Fig. 2A). These observations suggest that growth is more severely affected in Col-0
466 than Ws plants after exposure to excess Cu, agreeing with an enhanced constitutive Cu
467 homeostasis in Ws plants (Fig. 3A and Table 3). In addition to the COPT family members
468 (Sancenón et al., 2003), several other transporters are involved in plant nutrient distributions
469 (Puig et al., 2007). Amongst these, HMA proteins are implicated in the transport of essential and
470 non-essential heavy metals (Andrés-Colás et al., 2006; Hussain et al., 2004; Kobayashi et al.,
471 2008; Morel et al., 2009; Park et al., 2012; Puig et al., 2007; Wong and Cobbett, 2009). In
472 *Arabidopsis*, this P_{1B}-ATPase family consists of eight members, divided into two groups
473 according to metal-substrate specificity associated to the valence of the transported cations.
474 Whereas HMA1-4 are transporters of the divalent cations Cd/zinc/cobalt/lead, HMA5-8 transport
475 monovalent Cu or silver. Therefore, the mobilization of essential Cu in the plant does not involve
476 the same HMA proteins as the mobilization of non-essential Cd. Furthermore, different HMA
477 transporters have specific subcellular locations and functions. For example, the plasma membrane

478 HMA5 protein is involved in Cu translocation from roots to shoots (Kobayashi et al., 2008) and
479 Cu compartmentalization and detoxification within roots (Andrés-Colás et al., 2006). After
480 studying 103 different accessions, Kobayashi et al. (2008) suggested that the variation in Cu
481 tolerance observed in *A. thaliana* is partially regulated by the capacity of root-to-shoot Cu
482 translocation, associated with the functional integrity of HMA5. However, the Ws accession was
483 not included in that study and should be investigated in future research.

484 The root-to-shoot translocation factor of Cu significantly decreased in plants exposed to Cd
485 (Fig. 2A) compared to those not exposed, pointing towards a Cd-induced decreased ability of
486 plants in this condition to translocate Cu, which leads to Cd-induced Cu deficiency-like responses
487 (Gayomba et al., 2013; Gielen et al., 2016, 2017). Nonetheless, at each time point, the Cu
488 translocation factor was significantly higher in Cd-exposed Ws versus Col-0 plants (Fig. 2A).
489 Gielen et al. (2016) observed that Cd-induced Cu deficiency-like responses could be alleviated by
490 supplying extra Cu to Cd-exposed *Arabidopsis* plants, resulting in a lower *HMA5* upregulation in
491 Cu-supplemented plants as compared to non-supplemented plants (Gielen et al., 2017).
492 Therefore, the significantly lower *HMA5* upregulation in leaves of Cd-exposed Ws as compared
493 to Col-0 plants after 72 h (Supplementary Fig. 4) indicates that these Cu deficiency-like
494 responses were less pronounced in Ws plants. This again supports our statement that Cu
495 homeostasis mechanisms are less disturbed in leaves of Cd-exposed Ws plants.

496 After 24 h of exposure, the root-to-shoot Cd translocation factor was significantly higher in
497 Ws than in Col-0 plants (Fig. 2B). Both HMA2 and HMA4 are known to mediate Cd
498 translocation in *A. thaliana* (Wong and Cobbett, 2009), whereas HMA3 is involved in Cd
499 sequestration in the vacuole (Morel et al., 2009). In addition, it was shown that the *HMA3* gene
500 bears a point mutation in the Col-0 accession, consequently encoding for a truncated protein

501 differing from the protein in Ws plants (Hussain et al., 2004; Morel et al., 2009). Although an
502 obvious candidate gene, Fischer et al. (2017) observed that *HMA3* is unlikely determining the
503 variation in Cd tolerance observed in different *Arabidopsis* accessions. However, the Ws
504 accession was not included in that study and therefore this difference in HMA3 function may still
505 account for some of the differences in the Cd translocation factor observed between both
506 accessions (Fig. 2B). Moreover, Park et al. (2012) hypothesized that the non-functional HMA3
507 results in a preference for the expression of *HMA4* over *HMA2* in Col-0 plants, suggesting that
508 the cooperation between HMA3 and HMA4 is relevant for Cd detoxification. These authors also
509 reported that whereas short-term exposure to Cd did not alter *HMA4* expression in Ws plants, it
510 induced *HMA4* overexpression in Col-0 plants (Park et al., 2012). This can explain the time-
511 associated alterations to the Cd concentrations in leaves of both accessions (Supplementary Table
512 3) and the apparent arrest in the Cd transport observed in Ws plants considering the decreased
513 translocation factor (Fig. 2B). Since metal sequestration and transport are important mechanisms
514 in metal tolerance, future research on the root-to-shoot Cd translocation and *HMA2-4* expression
515 patterns after long-term exposure to Cd are required to further elucidate how Col-0 and Ws plants
516 cope with this toxic metal.

517 Exposure to excess Cu and Cd affected transcript levels of different genes in the leaves of both
518 accessions, as evidenced by the representation in heat maps (Fig.s 3B and 3C). Regardless of the
519 time point or accession, samples obtained from Cu- or Cd-exposed plants generally clustered
520 away from samples of non-exposed plants, allowing the identification of genes affected by each
521 metal. The heat map representation revealed that excess Cu affected genes involved in Cu
522 homeostasis mechanisms (Fig. 3B). These genes are known to be mediated by the central
523 regulator SQUAMOSA promoter binding protein-like 7 (SPL7) by way of its binding to GTAC

524 motifs within their promoter regions (Gayomba et al., 2013; Gielen et al., 2016; Yamasaki et al.,
525 2009). Although more research is needed, Dąbrowska et al. (2012) identified GTAC motif-
526 containing Cu response elements located in the promoter regions of *A. thaliana MT1a* and *MT1c*
527 genes, suggesting that these cysteine-rich proteins are also targeted by the SPL7 transcription
528 factor (Yamasaki et al., 2009). Therefore, the significant downregulation of *MT1c* after 24 h and
529 72 h of exposure and the upregulation of *MT2b* and *MT3* after 72 h suggest yet again that Ws
530 plants are more efficient at counteracting the altered Cu homeostasis. The same heat map
531 revealed another gene cluster including oxidative stress- and MAPK/ethylene signaling-related
532 genes, with a higher expression after exposure to excess Cu than under non-exposed conditions
533 (Fig. 3B). In particular, excess Cu appeared to induce different signaling mechanisms in leaves of
534 Col-0 and Ws plants (Table 4). The upregulation of *OX11* and *MPK3* suggests that MAPK
535 signaling pathways were activated in leaves of Cu-exposed Col-0 plants. The OX11 kinase is
536 essential to ROS sensing and MAPK signaling, linking oxidative burst signals to its downstream
537 responses (Rentel et al., 2004), such as the activation of detoxification mechanisms following Cu
538 exposure (Smeets et al., 2013). On the other hand, the upregulation of *MPK3*, *WRKY33* and
539 *ERF1* indicates that excess Cu stimulated ethylene signaling in leaves of Ws plants (Table 4).
540 The phytohormone ethylene regulates several developmental and physiological processes such as
541 seed germination, growth, flowering and senescence (Iqbal et al., 2017). Ethylene is also a known
542 “stress hormone”, modulating hormone and redox signaling processes under several biotic and
543 abiotic stress conditions including metal stress (Keunen et al., 2016; Schellingen et al., 2015a,
544 2015b) via a signaling cascade that, amongst others, induces the expression of *ERF1* (Huang et
545 al., 2016). Both MPK3 and MPK6 are known to play a role in controlling the rate-limiting step in
546 ethylene biosynthesis via the transcription factor WRKY33 (Li et al., 2012). Although ethylene
547 signaling is clearly favored in Cu-exposed Ws plants, the upregulation of *ERF1* in combination

548 with the upregulation of *OXI1* suggests that these two signaling-related molecules interact in
549 leaves of Col-0 plants in response to excess Cu. This interaction needs to be further investigated
550 to not only clarify the crosstalk between ethylene and ROS signaling, but also to explore the
551 activation of these signaling pathways in both accessions, particularly their time-related patterns.

552 Exposure to Cd increased the transcript levels of oxidative stress hallmark genes (Gadjev et
553 al., 2006), *LOX* genes and genes related to MAPK/ethylene signaling compared to non-exposed
554 plants (Fig. 3C). The overall higher upregulation of the oxidative stress hallmark genes and the
555 expression patterns of *LOX1* and *LOX2* in leaves of Cd-exposed Col-0 plants (Table 5) suggest
556 that these plants respond more strongly to Cd-induced oxidative stress than Ws plants. The
557 observed alterations in the GSH metabolism in leaves of Cd-exposed Col-0 plants, such as the
558 significant upregulation of *GSH2* (Table 5) and the increased GSH concentration (Table 2), point
559 towards the activation of detoxification mechanisms, by means of phytochelatin production, to
560 counteract the more severe Cd-induced oxidative stress response (Table 5). This is in agreement
561 with the proposed strategy favored by Col-0 plants. Several studies suggest an association
562 between GSH metabolism and ethylene signaling in metal stress conditions (Keunen et al., 2016;
563 Schellingen et al., 2015a, 2015b; Zhang et al., 2014), which appears to be supported by the
564 concurrent induction of both processes in the leaves of Cd-exposed Col-0 plants (Table 5).
565 Recently, Schellingen et al. (2015a) proposed a model linking ethylene biosynthesis, signal
566 transduction and oxidative stress in leaves of Cd-exposed *A. thaliana* leaves. In this model, it is
567 hypothesized that Cd induces an oxidative burst that leads to ethylene signaling via a MAPK
568 cascade initiated by *OXI1*. In turn, the ethylene signal cascade induces the expression of
569 downstream transcription factors such as *ERF1*. The expression of *ERF1* is known to increase in
570 response to ethylene signaling during Cd exposure (Schellingen et al., 2015a, 2015b). Moreover,

571 Schellingen et al., (2015a) also described ethylene to be involved in regulating GSH levels during
572 the early Cd-induced oxidative challenge. Indeed, several MAPK/ethylene signaling-related
573 genes were upregulated in leaves of Cd-exposed Col-0 plants, particularly after 24 h (Table 5),
574 suggesting a stronger ethylene signaling response in Col-0 than in Ws plants, which in turn might
575 have determined the GSH metabolism response observed in leaves of Col-0 plants (Tables 2 and
576 5).

577 In conclusion, our results suggest that Col-0 and Ws plants developed different life strategies.
578 While Ws plants have enhanced nutrient homeostatic capacities, particularly related to Cu
579 homeostasis mechanisms, Col-0 plants have boosted oxidative stress-related responses, mainly
580 related to MAPK/ethylene signaling and GSH detoxification mechanisms. This is evident not
581 only under non-exposed conditions, but also determines how both accessions respond to excess
582 Cu and Cd.

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