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Original paper

Accession-specific life strategies affect responses in leaves of *Arabidopsis thaliana* plants exposed to excess Cu and Cd

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Summary

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

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

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

1. Introduction

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

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

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

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

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

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

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

2. Material and methods

2.1. Plant material

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

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

2.2. Determination of metal concentrations

Leaf samples were first rinsed in distilled water and then dried at 60 °C for at least one week. Dried leaf samples were digested in HNO_3 (65 %) in a microwave oven (CEM MDS-2000 Microwave Digestor Oven, CEM Corporation, NC, USA), followed by a five-fold dilution in 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 KH_2PO_4 (pH 7.8) buffer, 0.1 mM EDTA, 10 µmM 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 (ε_{H2O2} = 40 mM^{-1} cm^{-1}) using 0.1 M KH_2PO_4 (pH 7.0) buffer
167 and 0.85 mM H_2O_2 (Bergmeyer et al., 1974). Guaiacol peroxidase (EC 1.11.1.7) activity was
168 determined at 436 nm ($\varepsilon_{tetraguaiacol}$ = 25.5 mM^{-1} cm^{-1}), in a reaction mixture containing a 0.1 M
169 KH_2PO_4 (pH 7.0) buffer, 0.8 mM H_2O_2 and 1.8 mM guaiacol (Bergmeyer et al., 1974).
170 Syringaldazine peroxidase (EC 1.11.1.7) activity was measured at 530 nm ($\varepsilon_{oxidized\ syringaldazine}$ =
171 11.6 mM^{-1} cm^{-1}), with the reaction mixture consisting of 0.1 M Tris-HCl (pH 7.5) buffer, 1 mM
172 H_2O_2, 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 (ε_{NADPH} = 6.22 mM^{-1} 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 $MnSO_4$, 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 $MgCl_2$ (Bergmeyer et al.,
182 1974).

2.4. Determination of glutathione concentrations

Oxidized (glutathione disulfide, GSSG) and reduced (GSH) forms of glutathione were analyzed on a plate reader by enzymatic assays based on the protocol described by Queval and Noctor (2007). Leaf samples were crushed in liquid nitrogen using a mortar and pestle, followed by homogenization in 200 mM HCl (pH 4.5). After reduction of GSSG to GSH by GR, total GSH and GSSG concentrations were determined by a kinetic enzymatic recycling assay based on the GSH-dependent reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The absorbance at 412 nm was measured in a 200 mM NaH_2PO_4 and 10 mM EDTA (pH 7.5) buffer containing 0.6 mM DTNB (in dimethyl sulfoxide), 0.5 mM NADPH and 1 U mL^{-1} GR. The rate of absorbance change over 5 min is proportional to the GSH concentration in the sample, which was calculated using GSH and GSSG standard curves ranging from 0 to 1 or 0.4 nmol, respectively. To determine the concentration of GSSG, the extracts (and GSSG standards) were first incubated in 1 % (V/V) 2-vinyl-pyridine (2-VP) at 20 °C for 30 min to derivatize GSH and then twice centrifuged for 10 min at 16 100 x *g* and 4 °C to precipitate 2-VP.

2.5. Gene expression analysis

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

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

2.6. Statistical analyses

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

3. Results

3.1. Rosette growth

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

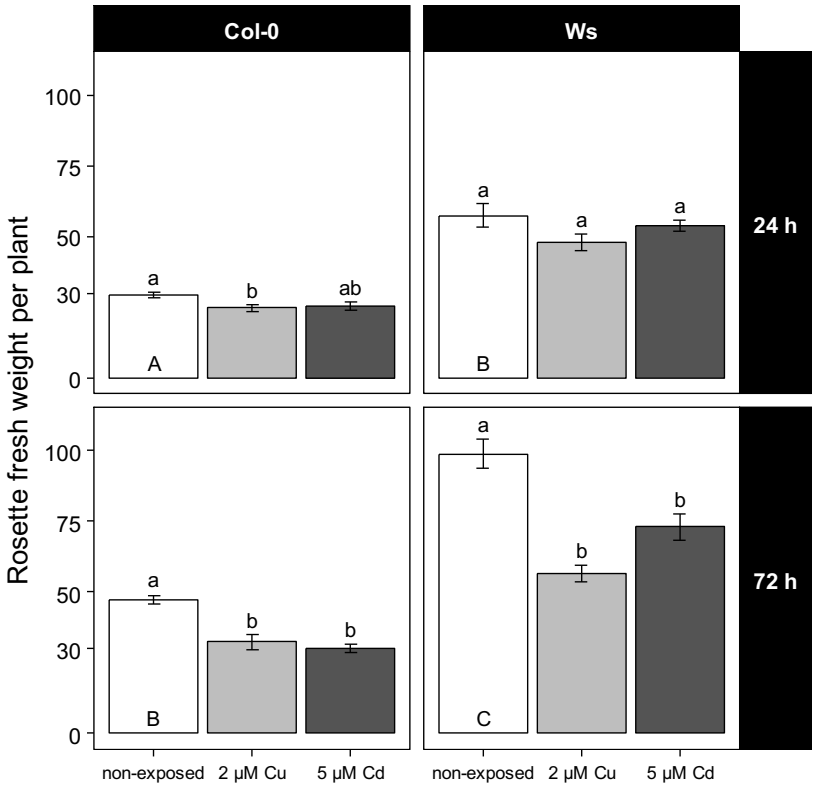

Fig. 1. Rosette fresh weight per plant (in mg) of three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws) exposed to 2 µM $CuSO_4$, 5 µM $CdSO_4$ or not exposed for 24 and 72 h. Values are mean ± S.E. of at least three biological replicates, each containing rosettes of 25 individual plants. □ = non-exposed. ■ = exposed to 2 µM $CuSO_4$. ■ = exposed to 5 µM $CdSO_4$. Statistical significance ($P < 0.05$) is indicated using lowercase (within accession and time point) or uppercase letters (between non-exposed accessions at both time points).

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

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

3.2. Metal translocation factors

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

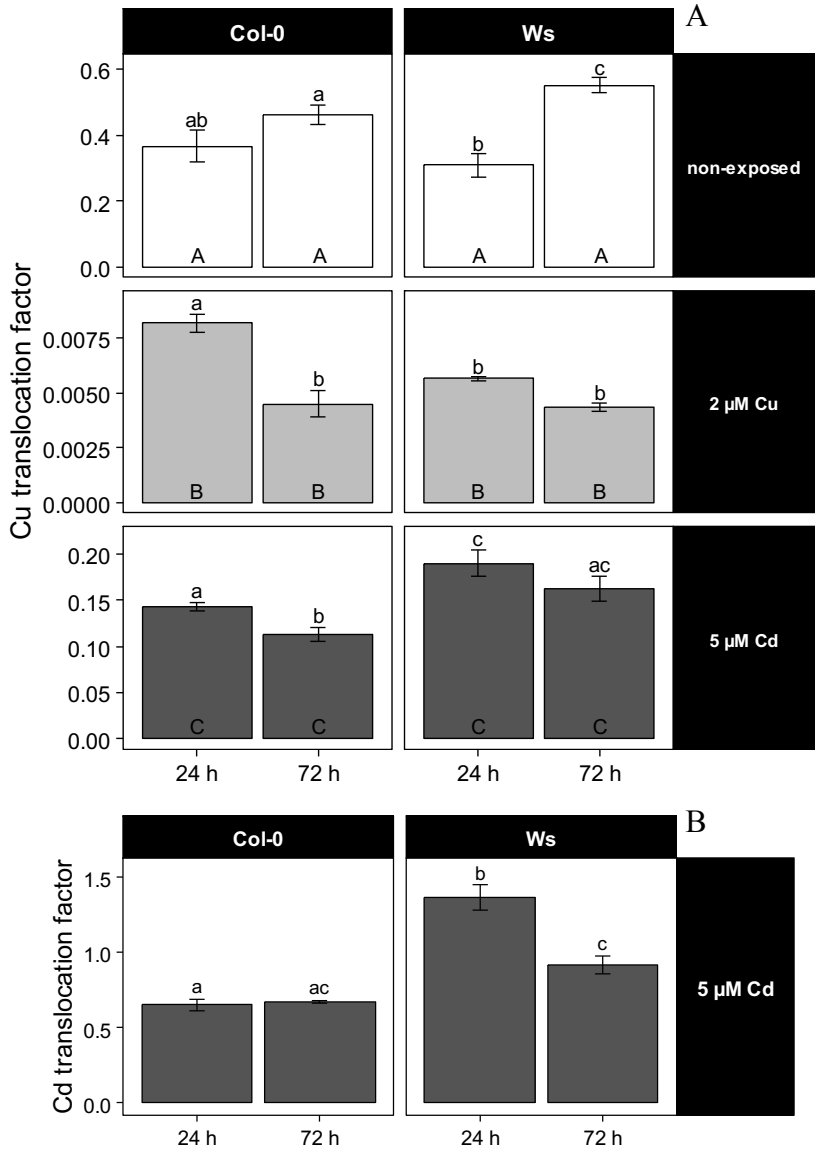

Fig. 2. Root-to-shoot translocation factors of Cu and Cd in three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws) exposed to 2 µM $CuSO_4$, 5 µM $CdSO_4$ or not exposed for 24 and 72 h. **(A)** Cu translocation factors in non-exposed, Cu- and Cd-exposed plants. **(B)** Cd translocation factors in Cd-exposed plants. Values are mean ± S.E. of at least three biological replicates. □ = non-exposed. ▒ = exposed to 2 µM $CuSO_4$. ■ = exposed to 5 µM $CdSO_4$. Statistical significance ($P < 0.05$) is indicated using different lowercase (within exposure condition, between accessions and time points) or uppercase letters (across exposure conditions, within accession and time point).

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

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

Table 1. Activities of antioxidative and redox-regulating enzymes (mU mg^{-1} fresh weight) in leaves of three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws) exposed to 2 µM $CuSO_4$, 5 µM $CdSO_4$ or not exposed for 24 and 72 h. Antioxidative enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD). NAD(P)H-producing enzymes: isocitrate dehydrogenase (ICDH), malic enzyme (ME) and glucose-6-phosphate dehydrogenase (G6PDH). Values are mean ± S.E. of at least three biological replicates, each containing rosettes of at least two individual plants. Statistical significance ($P < 0.05$) is indicated using different lowercase 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
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

3.4. Glutathione concentrations

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

Table 2. Concentrations of total (GSH + GSSG), reduced (GSH), oxidized (GSSG) glutathione (nmoles g^{-1} fresh weight)and GSSG/GSH ratio in leaves of three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws), exposed to 2 µM $CuSO_4$, 5 µM $CdSO_4$ or not exposed for 24 and 72 h. Values are mean ± S.E. of at least three biological replicates, each containing rosettes of at least one individual plant. Statistical significance ($P < 0.05$) is indicated using different lowercase 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

3.5. Gene expression

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

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

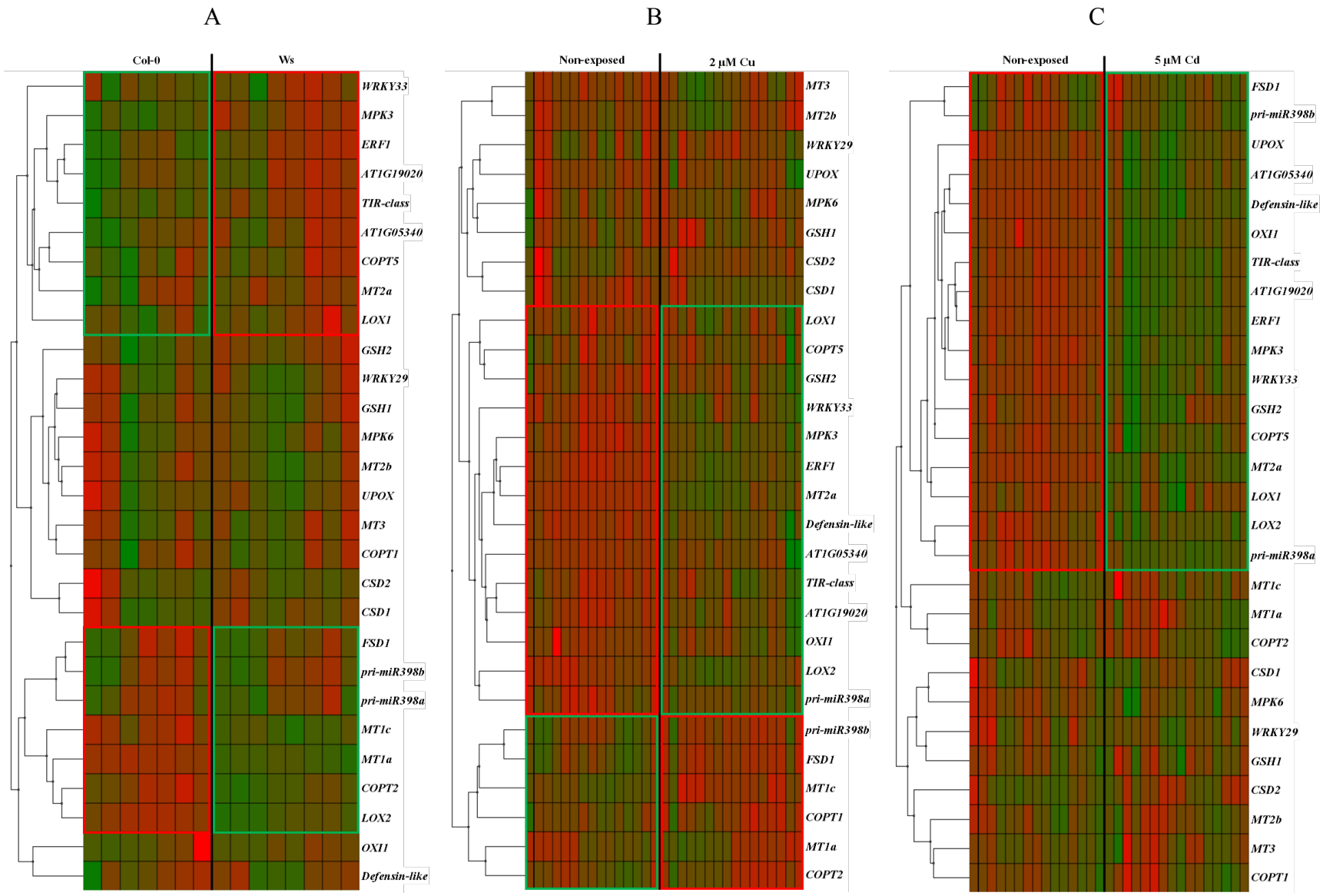
A
Col-0
Ws
WRKY33
MPK3
ERF1
AT1G19020
TIR-class
AT1G05340
COPT5
MT2a
LOX1
GSH2
WRKY29
GSH1
MPK6
MT2b
UPOX
MT3
COPT1
CSD2
CSD1
FSD1
pri-miR398b
pri-miR398a
MT1c
MT1a
COPT2
LOX2
OXI1
Defensin-like
B
Non-exposed
2 µM Cu
MT3
MT2b
WRKY29
UPOX
MPK6
GSH1
CSD2
CSD1
LOX1
COPT5
GSH2
WRKY33
MPK3
ERF1
MT2a
Defensin-like
AT1G05340
TIR-class
AT1G19020
OXI1
LOX2
pri-miR398a
pri-miR398b
FSD1
MT1c
COPT1
MT1a
COPT2
C
Non-exposed
5 µM Cd
FSD1
pri-miR398b
UPOX
AT1G05340
Defensin-like
OXI1
TIR-class
AT1G19020
ERF1
MPK3
WRKY33
GSH2
COPT5
MT2a
LOX1
LOX2
pri-miR398a
MT1c
MT1a
COPT2
CSD1
MPK6
WRKY29
GSH1
CSD2
MT2b
MT3
COPT1

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Fig. 3. Heat map representations of the gene expression data obtained in leaves of three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws). (A) Heat map of data collected from plants grown in non-exposed conditions for 24 and 72 h. (B) Heat map of data collected from plants exposed to 2 μM $CuSO_4$ or not exposed (C) Heat map of data collected from plants exposed to 5 μM $CdSO_4$ or not exposed. Hierarchical clustering of genes is shown at the top (gene names at the bottom). Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. Abbreviations: *UPOX: upregulated by oxidative stress; Defensin-like: protein member of the defensin-like (DEFL) family; AT1G19020: unknown protein; AT1G05340: unknown protein; TIR-class: Toll-Interleukin-Resistance (TIR) domain family protein; LOX1: lipoxygenase 1; GSH1: glutamate-cysteine ligase; GSH2: glutathione synthetase 2; CSD: Cu/Zn superoxide dismutase; FSD1: Fe superoxide dismutase 1; pri-miR398a: primary microRNA 398a; pri-miR398b: primary microRNA 398b; COPT: copper transporter; MT: metallothionein; OXI1: Oxidative signal inducible 1; MPK: mitogen-activated protein kinase; WRKY: WRKY DNA-binding protein; ERF1: ethylene response factor 1.*

Comparing non-exposed to Cu- or Cd-exposed samples revealed a separate clustering between the metal-exposed and non-exposed groups (Figs. 3B and 3C; for more detail see Supplementary Figs. 2 and 3). After including only non-exposed and Cu-exposed samples in the heat map, two groups of genes emerged (Fig. 3B): (1) as indicated by the red-shaded rectangles, genes that were less expressed after Cu exposure, including those involved in Cu homeostasis, such as *FSD1*, *pri-miR398b*, *COPT1*, *COPT2* and *MT1c* (Cluster Cu-I); (2) as indicated by the green-shaded rectangles, genes that were more expressed under excess Cu conditions, including those involved in MAPK/ethylene signaling, such as *OXI1*, *MPK3*, *WRKY33* and *ERF1*, four oxidative stress hallmark genes, *pri-miR398a*, *LOX1* and *LOX2*, *GSH2* and *MT2a* (Cluster Cu-II). Moreover, gene expression data revealed some accession-specific changes in the expression of these genes upon exposure to excess Cu (Table 4). Concerning the genes in cluster Cu-I, transcript levels of *FSD1*, *pri-miR398b* and *COPT2* were significantly lower only in leaves of Cu-exposed Ws plants after 72 h (Table 4). Expression of *MT1c* was significantly lower in leaves of Ws plants exposed to excess Cu at both time points (Table 4). Regarding the genes in cluster Cu-II, transcript levels of *GSH2* and *OXI1* were significantly higher only in leaves of Cu-exposed Col-0 plants (at 72 and 24 h respectively) (Table 4). Moreover, while *pri-miR398a* transcript levels were higher after

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

Leaves of Cd-exposed Col-0 and Ws plants generally clustered away from leaves of plants grown under non-exposed conditions (Fig. 3C). Several genes grouped together due to their higher expression upon Cd exposure (green-shaded rectangles). This cluster included oxidative stress-related genes such as all five oxidative stress hallmark genes, *GSH2*, *LOX1* and *LOX2*, the MAPK/ethylene signaling-related genes (*OXI1*, *MPK3*, *WRKY33* and *ERF1*), *COPT5*, *FSD1*, *MT2a* and *pri-miR398a/b* (Fig. 3C). Moreover, gene expression analysis revealed some accession-specific differences in their expression upon Cd exposure (Table 5). Transcript levels of oxidative stress hallmark genes (Gadjev et al., 2006) and ethylene signaling-related gene *ERF1* were generally more upregulated in leaves of Col-0 than Ws plants after Cd exposure (Table 5). Transcript levels of *LOX1* were significantly higher in leaves of Col-0 plants after 72 h exposure to Cd. While *LOX2* and *pri-miR398a* were upregulated in the leaves of both accessions after 24 h, their transcript levels were significantly higher in Col-0 than in Ws plants and remained upregulated after 72 h in the former accession (Table 5). Cadmium-induced upregulation of the *GSH2* gene occurred in leaves of Col-0 plants only. Exposure to Cd led to significantly increased expression levels of *MT2a* in leaves of both accessions after 72 h. Although *OXI1* expression increased in leaves of both accessions exposed to Cd, its transcript levels were significantly higher in leaves of Col-0 than Ws plants after 24 h (Table 5).

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

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

Col-0		**Non-exposed**	**Ws**	
24 h	**72 h**		**24 h**	**72 h**
1.00 ± 0.18	1.48 ± 0.17	Resolution	1.08 ± 0.10	1.40 ± 0.19
Genes encoding oxidative stress hallmark proteins				
1.00 ± 0.13	3.77 ± 1.28	*AT1G19020*	**0.51 ± 0.04 ***	1.86 ± 0.22
1.00 ± 0.13	1.73 ± 0.38	*AT1G05340*	0.86 ± 0.06	1.29 ± 0.09
1.00 ± 0.38	3.88 ± 2.41	*TIR-class*	**0.18 ± 0.03 ***	0.66 ± 0.21
Genes encoding ROS-producing enzymes				
1.00 ± 0.22	1.22 ± 0.15	*LOX1*	0.70 ± 0.09	1.14 ± 0.11
1.00 ± 0.17	**2.14 ± 0.43**	*LOX2*	**2.47 ± 0.20 ***	**4.85 ± 0.38 ***
Gene encoding antioxidative enzyme				
1.00 ± 0.48	15.09 ± 8.20	*FSD1*	2.24 ± 0.82	15.21 ± 3.04
Primary microRNA transcripts				
1.00 ± 0.22	46.45 ± 28.43	*pri-miR398a*	**3.97 ± 0.17 ***	94.66 ± 25.62
1.00 ± 0.22	15.31 ± 7.69	*pri-miR398b*	0.92 ± 0.21	11.75 ± 2.25
Genes encoding copper transporters				
1.00 ± 0.08	**2.58 ± 0.98**	*COPT2*	**3.16 ± 0.37 ***	**7.09 ± 0.48 ***
1.00 ± 0.10	1.28 ± 0.27	*COPT5*	0.74 ± 0.06	1.09 ± 0.05
Genes encoding metallothioneins				
1.00 ± 0.21	**1.81 ± 0.13**	*MT1a*	**5.46 ± 0.82 ***	**9.94 ± 2.87 ***
1.00 ± 0.02	**1.38 ± 0.01**	*MT1c*	**2.13 ± 0.24 ***	**2.36 ± 0.30 ***
1.00 ± 0.08	1.65 ± 0.32	*MT2a*	1.01 ± 0.07	1.37 ± 0.19
Gene encoding protein kinase				
1.00 ± 0.22	1.41 ± 0.35	*MPK3*	**0.53 ± 0.02 ***	0.75 ± 0.10
Gene encoding transcription factor				
1.00 ± 0.25	1.94 ± 0.62	*WRKY33*	0.55 ± 0.04	1.48 ± 0.21
Gene encoding protein involved in ethylene signaling				
1.00 ± 0.13	**4.38 ± 1.03**	*ERF1*	**0.49 ± 0.05 ***	**1.68 ± 0.19 ***

Table 4. Relative gene expression levels in leaves of three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws), exposed to 2 µM $CuSO_4$ for 24 and 72 h. Values are mean normalized expression relative to the non-exposed accession at each time point (set at 1.00) ± S.E. of at least three biological replicates, each containing rosettes of at least one individual plant. Resolution values are mean inverse normalization factors relative to the non-exposed accession at each time point, indicating the stability of the selected reference genes. Statistically significant ($P < 0.05$) metal-induced changes in expression relative to the non-exposed accession at each time point are indicated by color (■ = upregulation; ■ = downregulation). Statistically significant ($P < 0.05$) differences between accessions and within metal exposure are indicated by asterisks and printed in black and bold. Abbreviations: see Fig. 3.

Col-0		**2 µM Cu**	**Ws**	
24 h	**72 h**		**24 h**	**72 h**
1.49 ± 0.35	0.95 ± 0.21	Resolution	1.4 ± 0.39	1.11 ± 0.29
Genes encoding oxidative stress hallmark proteins				
1.38 ± 0.09	3.98 ± 1.49	*UPOX*	1.06 ± 0.00	2.02 ± 0.64
5.31 ± 0.53	7.86 ± 5.90	*Defensin-like*	3.99 ± 0.90	4.03 ± 0.52
6.07 ± 1.14	3.90 ± 1.77	*AT1G19020*	5.29 ± 1.77	3.40 ± 1.10
2.94 ± 0.48	8.97 ± 4.54	*AT1G05340*	3.95 ± 0.88	1.98 ± 0.24
16.6 ± 3.71	4.35 ± 2.04	*TIR-class*	16.03 ± 6.58	2.92 ± 1.27
Genes encoding ROS-producing enzymes				
1.42 ± 0.06	1.29 ± 0.23	*LOX1*	2.22 ± 0.24	1.41 ± 0.30
9.89 ± 0.45	2.70 ± 0.60	*LOX2*	5.33 ± 0.65	2.44 ± 0.55
Genes encoding antioxidative enzymes				
1.32 ± 0.10	**1.06 ± 0.05**	*GSH1*	1.24 ± 0.05	**0.80 ± 0.05 ***
1.39 ± 0.12	1.65 ± 0.11	*GSH2*	1.37 ± 0.06	1.62 ± 0.11
1.73 ± 0.18	2.28 ± 0.35	*CSD1*	2.34 ± 0.21	1.81 ± 0.60
0.96 ± 0.19	1.37 ± 0.05	*CSD2*	0.88 ± 0.09	1.04 ± 0.03
0.36 ± 0.07	0.07 ± 0.06	*FSD1*	0.21 ± 0.07	0.17 ± 0.15
Primary microRNA transcripts				
85.67 ± 23.78	5.40 ± 3.50	*pri-miR398a*	**12.1 ± 2.84 ***	1.82 ± 1.15
0.68 ± 0.13	0.25 ± 0.21	*pri-miR398b*	1.48 ± 0.50	0.08 ± 0.02
Genes encoding copper transporters				
0.90 ± 0.06	0.73 ± 0.05	*COPT1*	1.15 ± 0.08	1.13 ± 0.07
0.90 ± 0.01	0.32 ± 0.03	*COPT2*	1.04 ± 0.11	0.66 ± 0.02
1.38 ± 0.13	1.11 ± 0.19	*COPT5*	1.77 ± 0.08	1.08 ± 0.11
Genes encoding metallothioneins				
1.91 ± 0.27	0.93 ± 0.26	*MT1a*	1.55 ± 0.03	1.67 ± 0.46
0.70 ± 0.14	0.45 ± 0.15	*MT1c*	0.25 ± 0.03	0.09 ± 0.01
3.56 ± 0.43	2.19 ± 0.37	*MT2a*	4.14 ± 0.45	3.7 ± 0.36
1.22 ± 0.08	1.17 ± 0.29	*MT2b*	**1.76 ± 0.07 ***	2.00 ± 0.25
1.23 ± 0.06	1.41 ± 0.41	*MT3*	**1.93 ± 0.12 ***	2.79 ± 0.05

Genes encoding protein kinases				
10.65 ± 3.20	12.83 ± 6.78	*OXI1*	2.22 ± 0.42	3.12 ± 1.54
2.77 ± 0.23	1.68 ± 0.18	*MPK3*	3.92 ± 0.58	3.37 ± 0.46
1.07 ± 0.04	1.24 ± 0.07	*MPK6*	1.26 ± 0.04	1.18 ± 0.13
Genes encoding transcription factors				
0.60 ± 0.14	2.12 ± 0.25	*WRKY29*	0.64 ± 0.13	1.28 ± 0.25
4.14 ± 1.21	4.32 ± 1.08	*WRKY33*	5.48 ± 1.62	3.10 ± 0.54
Gene encoding protein involved in ethylene signaling				
24.71 ± 2.85	**4.76 ± 1.20**	*ERF1*	**86.51 ± 20.55 ***	**18.85 ± 5.43 ***

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Table 5. Relative gene expression levels in leaves of three-weeks-old *A. thaliana* plants (accessions Col-0 and Ws), exposed to 5 µM $CdSO_4$ for 24 and 72 h. Values are mean normalized expression relative to the non-exposed accession at each time point (set at 1.00) ± S.E. of at least three biological replicates, each containing rosettes of at least one individual plant. Resolution values are mean inverse normalization factors relative to the non-exposed accession at each time point, indicating the stability of the selected reference genes. Statistically significant ($P < 0.05$) metal-induced changes in expression relative to the non-exposed accession at each time point are indicated by color (■ = upregulation; ■ = downregulation). Statistically significant ($P < 0.05$) differences between accessions and within metal exposure are indicated by asterisks and 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 ± 0.22	0.74 ± 0.15		1.07 ± 0.16	1.01 ± 0.16
Genes encoding oxidative stress hallmark proteins				
5.93 ± 1.16	6.27 ± 2.19	*UPOX*	2.21 ± 0.19	2.21 ± 0.32
27.80 ± 4.70	46.09 ± 17.38	*Defensin-like*	**6.81 ± 0.60 ***	8.93 ± 2.33
129.71 ± 43.01	9.28 ± 4.87	*AT1G19020*	56.87 ± 15.44	3.58 ± 0.06
77.41 ± 36.40	30.25 ± 15.94	*AT1G05340*	7.82 ± 2.30	4.14 ± 1.55
231.62 ± 80.13	9.90 ± 4.53	*TIR-class*	422.48 ± 106.43	8.84 ± 1.00
Genes encoding ROS-producing enzymes				
1.99 ± 0.37	1.90 ± 0.18	*LOX1*	1.27 ± 0.04	1.11 ± 0.11
9.42 ± 2.21	2.30 ± 0.50	*LOX2*	**2.24 ± 0.08 ***	1.95 ± 0.51
Genes encoding antioxidative enzymes				
1.19 ± 0.19	**1.27 ± 0.07**	*GSH1*	0.92 ± 0.05	**0.94 ± 0.09 ***
3.31 ± 0.35	2.05 ± 0.27	*GSH2*	1.25 ± 0.07	1.46 ± 0.10
1.40 ± 0.23	0.85 ± 0.02	*CSD1*	1.25 ± 0.14	0.67 ± 0.06
0.61 ± 0.17	0.22 ± 0.01	*CSD2*	0.80 ± 0.08	0.45 ± 0.22
4.83 ± 1.14	0.85 ± 0.15	*FSD1*	1.92 ± 0.60	0.55 ± 0.28
Primary microRNA transcripts				
2330.58 ± 267.36	13.67 ± 0.86	*pri-miR398a*	**831.04 ± 180.57 ***	5.22 ± 2.00
7.51 ± 0.39	1.72 ± 0.15	*pri-miR398b*	5.00 ± 0.48	2.05 ± 0.10
Genes encoding copper transporters				
0.88 ± 0.09	0.88 ± 0.02	*COPT1*	1.12 ± 0.08	1.16 ± 0.10
1.41 ± 0.19	1.15 ± 0.27	*COPT2*	1.47 ± 0.05	1.38 ± 0.30
5.40 ± 1.06	1.28 ± 0.10	*COPT5*	2.76 ± 0.17	1.24 ± 0.16
Genes encoding metallothioneins				
1.07 ± 0.27	0.45 ± 0.16	*MT1a*	0.93 ± 0.11	1.22 ± 0.08
1.03 ± 0.06	1.70 ± 0.25	*MT1c*	0.65 ± 0.09	0.61 ± 0.22
3.30 ± 0.44	2.22 ± 0.22	*MT2a*	2.21 ± 0.06	2.63 ± 0.17
0.93 ± 0.09	0.88 ± 0.08	*MT2b*	0.94 ± 0.05	1.32 ± 0.21
0.74 ± 0.12	0.88 ± 0.07	*MT3*	0.94 ± 0.14	1.30 ± 0.34

Genes encoding protein kinases				
272.11 ± 75.02	62.59 ± 36.07	*OXI1*	**38.74 ± 1.32 ***	5.94 ± 1.96
5.83 ± 1.49	2.24 ± 0.34	*MPK3*	5.35 ± 0.61	2.34 ± 0.38
2.28 ± 0.51	1.40 ± 0.06	*MPK6*	1.48 ± 0.12	1.11 ± 0.09
Genes encoding transcription factors				
1.02 ± 0.07	2.80 ± 0.33	*WRKY29*	0.93 ± 0.03	1.53 ± 0.32
41.42 ± 13.28	7.69 ± 3.75	*WRKY33*	18.64 ± 5.94	2.82 ± 0.29
Gene encoding protein involved in ethylene signaling				
759.48 ± 165.90	13.11 ± 4.95	*ERF1*	289.41 ± 46.10	12.64 ± 2.50

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4. Discussion

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

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

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

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

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

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

motifs within their promoter regions (Gayomba et al., 2013; Gielen et al., 2016; Yamasaki et al., 2009). Although more research is needed, Dąbrowska et al. (2012) identified GTAC motif-containing Cu response elements located in the promoter regions of *A. thaliana MT1a* and *MT1c* genes, suggesting that these cysteine-rich proteins are also targeted by the SPL7 transcription factor (Yamasaki et al., 2009). Therefore, the significant downregulation of *MT1c* after 24 h and 72 h of exposure and the upregulation of *MT2b* and *MT3* after 72 h suggest yet again that Ws plants are more efficient at counteracting the altered Cu homeostasis. The same heat map revealed another gene cluster including oxidative stress- and MAPK/ethylene signaling-related genes, with a higher expression after exposure to excess Cu than under non-exposed conditions (Fig. 3B). In particular, excess Cu appeared to induce different signaling mechanisms in leaves of Col-0 and Ws plants (Table 4). The upregulation of *OXI1* and *MPK3* suggests that MAPK signaling pathways were activated in leaves of Cu-exposed Col-0 plants. The OXI1 kinase is essential to ROS sensing and MAPK signaling, linking oxidative burst signals to its downstream responses (Rentel et al., 2004), such as the activation of detoxification mechanisms following Cu exposure (Smeets et al., 2013). On the other hand, the upregulation of *MPK3*, *WRKY33* and *ERF1* indicates that excess Cu stimulated ethylene signaling in leaves of Ws plants (Table 4). The phytohormone ethylene regulates several developmental and physiological processes such as seed germination, growth, flowering and senescence (Iqbal et al., 2017). Ethylene is also a known "stress hormone", modulating hormone and redox signaling processes under several biotic and abiotic stress conditions including metal stress (Keunen et al., 2016; Schellingen et al., 2015a, 2015b) via a signaling cascade that, amongst others, induces the expression of *ERF1* (Huang et al., 2016). Both MPK3 and MPK6 are known to play a role in controlling the rate-limiting step in ethylene biosynthesis via the transcription factor WRKY33 (Li et al., 2012). Although ethylene 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,

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

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

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