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Correlation between hormonal homeostasis and morphogenic responses in *Arabidopsis thaliana* seedlings growing in a Cd/Cu/Zn multi-pollution context

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To date, almost no information is available in roots and shoots of the model plant *Arabidopsis thaliana* about the hierarchic relationship between metal accumulation, phytohormone levels, and glutathione/phytochelatin content, and how this relation affects root development. For this purpose, specific concentrations of cadmium, copper and zinc, alone or in triple combination, were supplied for 12 days to in vitro growing seedlings. The accumulation of these metals was measured in roots and shoots, and a significant competition in metal uptake was observed. Microscopic analyses revealed that root morphology was affected by metal exposure, and that the levels of *trans*-zeatin riboside, dihydrozeatin riboside, indole-3-acetic acid and the auxin/cytokinin ratio varied accordingly. By contrast, under metal treatments, minor modifications in gibberellic acid and abscisic acid levels occurred. Real-time polymerase chain reaction analysis of some genes involved in auxin and cytokinin synthesis (e.g. *AtNIT* in roots and *AtIPT* in shoots) showed on average a metal up-regulated transcription. The production of thiol-peptides was induced by all the metals, alone or in combination, and the expression of the genes involved in thiol-peptide synthesis (*AtGSH1*, *AtGSH2*, *AtPCS1* and *AtPCS2*) was not stimulated by the metals, suggesting a full post-transcriptional control. Results show that the Cd/Cu/Zn-induced changes in root morphology are caused by a hormonal unbalance, mainly governed by the auxin/cytokinin ratio.

Introduction

Phytoremediation is an important tool for removing metal(loid)s and other contaminants from polluted sites (Vangronsveld et al. 2009). Real progress in the area depends on an in depth-analysis of the plant responding mechanisms to metal action, both at molecular and cellular levels (Hassan and Aarts 2011). Understanding the mechanisms of plant perception and transduction of metal-induced stress to initiate acclimation or adaptive

Abbreviations – AAO, aldehyde oxidase; ABA, abscisic acid; *Arabidopsis*, *Arabidopsis thaliana*; AUX, auxin; CK, cytokinin; CKX, cytokinin oxidase; DW, dry weight; DHZR, dihydrozeatin riboside; GSH, reduced glutathione; IAA, indole-3-acetic acid; IPT, isopentenyl transferase; NIT, nitrilase; PC, phytochelatin; PCS, phytochelatin synthase; PCs, phytochelatins; *t*-ZR, *trans*-zeatin riboside.

responses is in fact essential for improving metal tolerance in crops and in phytoremediation-addressed plants.

Cadmium (Cd), copper (Cu) and zinc (Zn) are common metals of polluted soils. In plants, Zn and Cu are essential for normal metabolism and growth, although an excessive, non-homeostatic, supply of these micronutrients can result into toxicity symptoms and altered morphogenesis (Kabata-Pendias and Mukherjee 2007). In fact, Zn and Cu tissue concentrations for normal growth and development of plants range from 30 to 200 $\mu g\,g^{-1}$ dry weight (DW) for Zn, and from 6 to 12 $\mu g\,g^{-1}$ DW for Cu (Marschner 2012). By contrast, a tissue concentration $\geq 400\,\mu g\,g^{-1}$ DW of Zn and 35 $\mu g\,g^{-1}$ DW of Cu is to be considered toxic for nearly all plants (Marschner 2012). A non-essential, toxic metal such as Cd (levels of 1–5 μM in the soil solution are sufficient to retard root growth; Sanità di Toppi and Gabbrielli 1999) is to be rendered less toxic by timely detoxification mechanisms (Cuypers et al. 2010).

Arabidopsis thaliana (*Arabidopsis*) is a model plant for many studies, as its genome sequence has been identified, and a large molecular knowledge on this species thus generated. Interestingly, the morphogenesis of *Arabidopsis* exposed to metal stress often resembles that of plants altered in phytohormone metabolism (Pasternak et al. 2005, Kai et al. 2007), particularly as far as roots are concerned (Brunetti et al. 2011). In *Arabidopsis*, key components of the signal-transduction pathways that affect auxin-dependent lateral root initiation have been identified (Casimiro et al. 2003, Aloni et al. 2006). It is known that indole-3-acetic acid (IAA), the main auxin (AUX) in plants, is involved in lateral root initiation and emergence, by causing changes in cell division, expansion and differentiation in a number of plants, including *Arabidopsis* (Aloni et al. 2006). Also, the natural cytokinins (CKs) *trans*-zeatin riboside (*t*-ZR) and dihydrozeatin riboside (DHZR) are implied in meristem activity, de novo bud formation, release from apical dominance and other morphophysiological events (Werner and Schmülling 2009). In primary and lateral roots, the balance between division and differentiation is governed by a crosstalk between AUXs and CKs (Jones and Ljung 2012). In *Arabidopsis*, a Cd-induced inhibitory action has also been reported to be concomitant with an increased endogenous abscisic acid (ABA) content, indicating that ABA takes part in the metal-imposed phytotoxicity (Sharma and Kumar 2002). In *Arabidopsis*, gibberellins (e.g. gibberellic acid, GA3) affect almost every event of plant growth and development, particularly stem and root growth, through cell elongation and increased expression of several genes (Yamaguchi 2006, Peleg and Blumwald 2011). Not least, other plant hormones, such as ethylene and jasmonic acid, appear to play a key role in the changes of growth processes mediated by metals (Maciejewska and Kopcewicz 2002, Maksymiec 2011). Considerable effort has also been directed in this species at clarifying processes, and factors, contributing to IAA, ABA, gibberellins and, in general, phytohormone integrated-roles and homeostasis in metal responses, but the entire picture is fragmentary and remains to be elucidated (Sofo et al. 2012).

Phytochelatins [PCs; general structure (γ-Glu-Cys) nGly] are synthesized from reduced glutathione (GSH) (γ-Glu-Cys-Gly) in a reaction catalyzed by the enzyme phytochelatin synthase (PCS), a γ-glutamylcysteine dipeptidyl transpeptidase (EC 2.3.2.15) (Cobbett 2000, Sanità di Toppi et al. 2003). PCs are a class of metal-binding thiol-peptides playing relevant roles in Cd, Cu, Zn detoxification, and represent a major metal detoxifying system for plants and some other organisms (Sanità di Toppi et al. 2003, Bolchi et al. 2011, Seth et al. 2012). The *Arabidopsis AtPCS1* (=*CAD1*) gene encodes for PCS1, so that *cad1* knockout mutants are PC deficient and Cd hypersensitive (Vatamaniuk et al. 1999, Peterson and Oliver 2006). The *Arabidopsis* genome also contains a highly homologous gene, *AtPCS2*, that encodes for a functional PCS apparently non-redundant with *AtPCS1* (Cazalé and Clemens 2001).

Tennstedt et al. (2009) suggested a contribution of *PCS1* expression, and PC accumulation, to Zn sequestration and homeostasis in *Arabidopsis* roots. Not least, the over-expression of *AtPCS1* in tobacco (Pomponi et al. 2006) and *Arabidopsis* (Brunetti et al. 2011) increases the production of PCs, leading to a general increment of plant Cd-tolerance when the metal is supplied at high concentrations. Finally, Wójcik et al. (2009) have found that Cu (ranging from 5 to 50 μM) does not induce PC accumulation nor significantly affect GSH level in *Arabidopsis*.

Based on this, the aim of this work was to investigate metal accumulation, morphological root modifications, and changes in the levels of various phytohormones and thiol-peptides, in relation to gene expression, in *Arabidopsis* seedlings exposed to Cd, Cu and Zn, supplied both alone and in triple combination.

Materials and methods

Plant material and experimental design

Seeds of *A. thaliana* (Columbia ecotype; Col-0) were sterilized using 50% (v/v) ethanol for 5 min followed by 5 min of 1% Na-hypochlorite, and finally rinsed with sterile dH_2O, before inhibition on moist filter paper

at 4°C for 24 h in the dark. Then, seeds were put in polyethylene containers (three containers per treatment and 36 seeds per container) filled with sterilized sand (mean particle diameter = 0.25 mm, in order to allow an efficacious and rapid root extraction from the sand, without tissue damage), and moistened with 300 ml of one-quarter strength Hoagland liquid medium. Throughout the experiment, the Hoagland solution was continuously replaced in order to maintain a constant volume of 300 ml and to keep the roots moistened. Seedlings were grown in a growth chamber at 20°C with a 16-h photoperiod and a photosynthetic photon flux density of 300 µmol m^{-2} s^{-1} at leaf level. Two-week-old seedlings were subsequently exposed to 10 µ*M* $CdSO_4$, 5 µ*M* $CuSO_4$, 150 µ*M* $ZnSO_4$, alone or in triple combination (Cd/Cu/Zn), simulating the concentrations possibly present in the soils contaminated by these three metals (Vangronsveld et al. 2009, Sofo et al. 2012). After 12 days of exposure to the metals, roots and shoots were separated and immediately analyzed.

Metal content measurements

Roots were carefully rinsed twice with 10 m*M* $CaCl_2$ and then with dH_2O, according to Lasat et al. (1996). Aliquots of shoots and roots (1 g) were digested in an HNO_3:H_2O_2 solution (5:1 v/v), by using a high performance microwave digestion unit (MLS-1200 Mega, Milestone Inc., CT). The levels of Cd, Cu and Zn were determined by inductively coupled plasma-atomic emission spectrometry (ICP-OES; model iCAP 6000, Thermo-Scientific, Cambridge, UK). Blanks (only HNO_3 and H_2O_2) and a standard stock solution of 50 µmol l^{-1} for each element were analyzed for reference purposes.

Growth observations

Shoot height (flowering stem), primary root length, and rosette diameter were measured on 20 randomly selected seedlings per treatment. Fresh root systems from 10 seedlings were immediately mounted on slides and observed using a microscope (Eclipse 80i, Nikon, Tokyo, Japan) under transmitted light, and then photographed (Digital Camera DS-Fi1, Nikon). Root hairs density (evaluated as hair number normalized to root length), root branching (number of lateral roots normalized to root length) and mean root diameter (measured at 0.02 cm from the tips of primary and lateral roots), were measured using the NIS-Elements Imaging Software (Nikon). Root branching or root hair calculated at the same position for each treatment.

Phytohormone and thiol-peptide analyses

Aliquots of 250 mg of shoot and root tissues were homogenized in a mortar ice in 2.5 ml of 2-propanol/H_2O/HCl 37% (2:1:0.002, v/v/v). To each sample, 2.5 ml of dichloromethane was added, and subsequently the samples were centrifuged at 13 000 *g* for 5 min. Subsequently, 1.0 ml of the solvent from the lower phase was concentrated using an evaporator with nitrogen flow, and then re-dissolved in 0.1 ml methanol. The quantitative determinations of IAA, *t*-ZR, DHZR, GA3 and ABA were carried out by high performance liquid chromatography coupled with mass spectrometry (Shimadzu LCMS-2020 equipped with an ESI source, with two LC-2020 AD pumps, CBM-20A controller and SIL-20A MS-2020 auto-sampler; Shimadzu Co., Kyoto, Japan), according to Sofo et al. (2011).

For thiol-peptides analysis, root and shoot samples from control and metal-exposed plants were homogenized in a mortar in ice-cold 5% (w/v) 5-sulfosalicylic acid, containing 6.3 m*M* diethylenetriaminepentaacetic acid, and analyzed as reported in Pomponi et al. (2006).

Total RNA extraction and real-time reverse transcription PCR (qRT-PCR)

Total RNA from *Arabidopsis* shoots and roots was extracted by TRIzol® Reagent (Invitrogen, Milan, Italy). Tissues (100 mg) deriving from control and metal-exposed seedlings were ground with mortar and pestle in 1 ml of sterile RNase-free water. To 300 µl of crude extract, 1 ml of TRIzol® Reagent was added. The sample was homogenized and the procedures for the dissociation of nucleoprotein complexes, phase separation, RNA precipitation, RNA washing and RNA re-dissolution were carried out as described by the manufacturer.

Priming of the cDNA reaction from the RNA template was carried out starting from an amount of 1 µg of total RNA extracted. The reaction was performed using the First-Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK), in a final volume of 15 µl, by using the random hexadeoxynucleotides $pd(N)_6$, according to the manufacturer. Primer pairs were designed for the real-time reverse transcription PCR amplification (qRT-PCR) of sequences belonging to genes known for having a regulatory role in the biochemical pathways of IAA, CKs, GSH and PCs (Srivastava 2002, Sanità di Toppi et al. 2003, Hedden and Thomas 2006, Miyawaki et al. 2006, Peleg and Blumwald 2011) (Supporting Information, Table S1). qRT-PCR was performed in an optical 96-well plate with a 7500 Fast sequence detection system (Applied Biosystems, CA) using 'fast mode' universal cycling

conditions, followed by the generation of a dissociation curve to check for specificity of amplification. Reactions contained Fast SYBR Green Master Mix (Applied Biosystems), 300 n*M* of a gene specific forward and reverse primer (Table S1) and 2 µl of the diluted cDNA in each reaction.

Relative expression for genes of interest in each sample was calculated as $2^{-\Delta Cq}$, and normalized by the geometric average of $2^{-\Delta Cq}$ values for three reference genes per sample. Reference genes At5g15710 (F-box protein), At2g28390 (SAND family protein) and At5g08290 (mitosis protein YLS8) for normalizing gene expression data when plants are exposed to excess metals were identified previously (Remans et al. 2008).

Statistics

The number of replicates (n) for each measured parameter is specified throughout the text, and in the table and figure captions. The statistical analysis of data was carried out using the SIGMASTAT 3.1 SPSS Inc. software (SPSS Inc., IL). Analysis of variance (ANOVA) of root and shoot parameters was performed with metal treatments as factors. Means were statistically analyzed by Fisher's LSD test at $P \leq 0.05$. qRT-PCR data were analyzed by one-way ANOVA with Tukey *post hoc* test ($P \leq 0.05$), after Shapiro–Wilk normality test. Detailed statistics is available in the supplementary material (Table S2 and Fig. S1).

Results

Metal accumulation and growth parameters

Seedlings exposed to metals for 12 days efficiently took the three metals up, which accumulated in roots and were partly translocated to shoots (Fig. 1A, B). The Cd root uptake was subjected to a significant competition in the presence of Cu and Zn, reducing of about fourfold its accumulation if compared to its content without the other two metals (Fig. 1A). A similar, but less marked behavior was observed for Cu levels in roots, with a 34% reduction in the Cd/Cu/Zn treatment, compared to the Cu alone. No decrease in Zn levels was found in the Cd/Cu/Zn treatment when compared to the content of Zn-exposed seedlings (Fig. 1A). The metals were found in lower concentrations in the shoots, but in similar ratios of those in the roots (Fig. 1B). In fact, despite the antagonism of metal uptake in the combined treatment, shoot translocation seems to have remained generally unaffected (Fig. 1B). Cd was the most translocated metal, as the shoot/root Cd ratio in the Cd treatment was 0.57 and it remained higher (0.61) in the triple treatment.

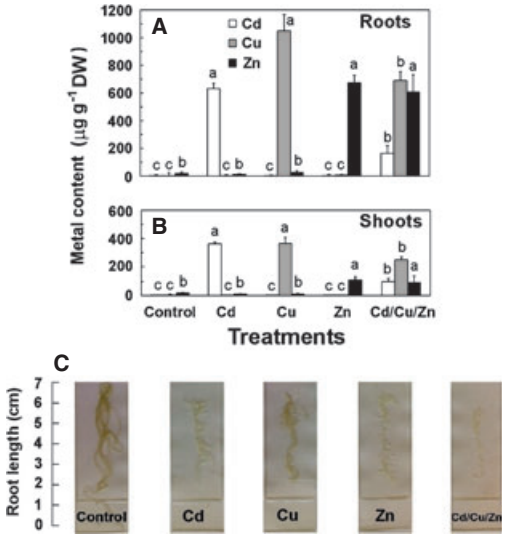

Fig. 1. Concentrations of Cd, Cu and Zn in roots (A; above) and shoots (B; below) of *Arabidopsis* seedlings not exposed to metals (Control), or exposed for 12 days to 10 µ*M* $CdSO_4$ (Cd), 5 µ*M* $CuSO_4$ (Cu), and 150 µ*M* $ZnSO_4$ (Zn), alone or in triple combination (Cd/Cu/Zn). Mean values (n = 10) ± SD with different letters for each metal are significantly different between treatments at $P \leq 0.05$, according to Fisher's LSD test. (C) Roots of *Arabidopsis* seedlings not exposed to metals (Control), or exposed for 12 days to 10 µ*M* $CdSO_4$ (Cd), 5 µ*M* $CuSO_4$ (Cu), and 150 µ*M* $ZnSO_4$ (Zn), alone or in triple combination (Cd/Cu/Zn). [Correction added on 19 June 2013, after first online publication: Figure legend 1(B) has been amended]

The shoot/root ratios for the treatments with the other two metals were equal to about 0.35 for Cu and 0.15 for Zn, both for the single and for the multi-exposure experimental set.

Shoot color turned from light green in the control to reddish-green in Cu- and Zn-exposed seedlings, to yellowish-green in Cd-exposed seedlings, whereas the seedlings subjected to Cd/Cu/Zn treatment appeared to be brownish-green. After 12 days of metal exposure, the seedlings showed macroscopic differences in root development, if compared to controls (Fig. 1C). Primary root length decreased in the presence of metals, showing mean values (± SD) (n = 20) of 7.4 (± 0.7), 4.2 (± 0.7), 4.3 (± 0.4), 4.9 (± 0.4) and 3.0 (± 0.3) cm for control, Cd, Cu, Zn and Cd/Cu/Zn treatments, respectively (Fig. 1C). The same behavior of primary root was observed for shoot height, whose mean values (± SD) (n = 20) were 6.4 (± 0.8), 5.0 (± 0.3), 5.2 (± 0.6), 5.5 (± 0.6) and 3.8 (± 0.2) cm for control, Cd, Cu, Zn and Cd/Cu/Zn treatments, respectively. Finally, metal application affected rosette diameter, a parameter that gives a reasonable idea of plant growth (Pasternak et al. 2005, Sofo et al. 2012), causing reductions from 3.5 (± 0.3) cm (mean values ± SD; n = 20) in the controls, to 2.4 (± 0.3),

2.7 (± 0.4), 2.9 (± 0.4) and 2.0 (± 0.2) cm in Cd, Cu, Zn and Cd/Cu/Zn treatments, respectively.

Phytohormone levels

The levels of IAA in the roots increased significantly up to more than fivefold in the Cd/Cu/Zn combination if compared to controls (at $P \leq 0.001$), whereas the IAA levels in the seedlings exposed to Cd and Cu alone were about two times higher than the control ($P \leq 0.01$) (Tables 1 and S2). By contrast, IAA levels in roots of Zn-exposed plants did not differ from those of control plants (Table 1). In the shoots, IAA levels were particularly low if compared to those detected in the roots, and significant variations attributable to metal exposure were not found (Tables 1 and S2).

The levels of the cytokinins *t*-ZR and DHZR significantly increased in the presence of single/multiple metal exposures, both in roots and shoots (Tables 1 and S2), except for DHZR in Cu-exposed roots. In general, in the shoots CK contents were high if compared to the roots, and showed to be particularly elevated in the triple metal treatment (Table 1). Besides, metal single exposure significantly increased ($P \leq 0.05$) the GA3 levels in the root (2.6-, 2.2- and 3.2-fold in the Cd, Cu and Zn treatments, respectively, compared to controls); however, this was not the case of the triple metal mixture (Tables 1 and S2). On the contrary, GA3 levels in the shoots did not show differences among the treatments (Tables 1 and S2). Finally, although ABA levels in metal-exposed roots showed an upward trend, significant increases due to metal exposure were never observed (Tables 1 and S2). In the shoots, the ABA levels were extremely low, except for the Zn-exposed plants, but in any case, its values did not significantly differ from the control plants.

Effects of Cd, Cu and Zn on root morphology

Microscopic observations revealed that root morphology was heavily affected by the exposure to the metals, both alone and in triple combination (Fig. 2 and Table S2). First, single or combined exposure to Cd, Cu, Zn lead to strong increases in root hairs density, if compared to controls ($P \leq 0.05$ for Cu and $P \leq 0.01$ for the other treatments) (Fig. 2A). Indeed, control seedlings presented a mean (± SD) ($n = 10$) root hair number of 5 (± 3) mm^{-1} root, whereas the values in the metal-exposed seedlings were 28 (± 7), 19 (± 8), 23 (± 7) and 21 (± 5) mm^{-1} root for Cd, Cu, Zn and Cd/Cu/Zn treatments, respectively. Second, Cd, Cu and Zn, alone or in combination, caused intense and significant increases in root branching ($P \leq 0.05$), when compared to controls, however, with no difference within them (Fig. 2B and Table S2). Compared to the control, the roots of the plants exposed to each of the three metals showed a significant increase in the mean diameter ($P \leq 0.05$), which was further enhanced by the triple combination ($P \leq 0.01$) (Fig. 2C and Table S2).

Thiol-peptide amounts

Following Cd, Zn and Cd/Cu/Zn exposure, GSH in roots underwent significant increases, compared with controls (at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.05$, respectively); by contrast, Cu treatment did not show to affect the root GSH content (Fig. 3A and Table S2). In the shoots, no significant variation in GSH levels was observed after the single metal exposures, whereas the triple treatment strongly increased GSH levels ($P \leq 0.01$) (Fig. 3B and Table S2).

PCs, which were not detectable in control plants, were detected in both organs of the metal-exposed plants, and (much) more abundantly, on average, in the roots than in the shoots (Fig. 3A, B). For both organs, Cd exposure caused strong significant increases of PCs (at $P \leq 0.001$ in roots and $P \leq 0.01$ in shoots), followed by Zn and then by Cu (Fig. 3A, B and Table S2). In roots, the combined metal exposure lead to higher PC contents than exposure to only Cu, although the levels were lower than in the case of exposure to only Cd or Zn (Fig. 3A). In the shoots, the PCs level induced by the combined metal exposure was not different from that induced by Cd alone, and higher than for the single Cu and Zn exposures (Fig. 3B). Under Zn stress, PC_3 was the most abundant oligomer in the roots at 130.78 nmol -SH g^{-1} fresh weight, whereas no particular PC isoform was predominant after Cd and Cu given alone or in the triple treatment (Fig. S2A). PC_4 and PC_5 were also detected in the roots (Fig. S2A). In the shoots, PC_2 and PC_3 always accumulated to a similar extent, whereas PC_4 and PC_5 were the less abundant oligomers, independently on the metal supplied (Fig. S2B).

Quantitative-PCR analysis

The expression of *NIT* gene, encoding for the enzyme nitrilase, catalyzing the final regulatory reaction of IAA biosynthetic pattern (Srivastava 2002, Hedden and Thomas 2006), was up-regulated in roots by the exposure to Cd and Cu (at $P \leq 0.01$ and $P \leq 0.05$, respectively) and by the three metals in combination (3.4-, 2.2- and 3.5-fold, respectively) ($P \leq 0.001$), whereas it showed a non-significant, downward trend given by Zn alone (0.5-fold) (Tables 2 and S2). In shoots, *NIT* expression did not change when plants were exposed to the metals,

Table 1. Levels of indole-3-acetic acid (IAA), trans-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR), gibberellic acid (GA3) and abscisic acid (ABA) in roots and shoots of *Arabidopsis* seedlings not exposed to metals (Control), or exposed for 12 days to 10 μ*M* $CdSO_4$ (Cd), 5 μ*M* $CuSO_4$ (Cu), and 150 μ*M* $ZnSO_4$ (Zn), alone or in combination (Cd/Cu/Zn). Mean values (n = 10) ± SD with different letters are significantly different between treatments at $P \leq 0.05$, according to Fisher's LSD test.

		IAA	*t*-ZR	DHZR	GA3	ABA
		(nmol g^{-1} FW)				
Roots	Control	157.29 ± 11.80 d	7.80 ± 0.44 c	8.20 ± 1.66 c	5.53 ± 0.35 b	3.02 ± 1.37 a
	Cd	276.70 ± 48.42 c	9.41 ± 0.75 b	10.14 ± 1.41 b	14.56 ± 1.89 a	3.88 ± 1.41 a
	Cu	273.15 ± 32.78 c	10.41 ± 0.64 b	8.32 ± 1.67 c	12.04 ± 0.60 a	4.63 ± 0.66 a
	Zn	137.50 ± 33.40 d	9.01 ± 0.30 b	9.17 ± 1.35 b	17.50 ± 2.31 a	5.75 ± 0.56 a
	Cd/Cu/Zn	820.00 ± 51.21 a	13.76 ± 0.21 a	13.94 ± 0.79 a	5.00 ± 1.55 b	5.00 ± 1.95 a
Shoots	Control	57.00 ± 2.28 a	24.62 ± 1.97 c	26.13 ± 2.65 d	1.60 ± 0.16 a	0.80 ± 0.15 a
	Cd	24.16 ± 3.14 a	61.17 ± 7.95 b	119.42 ± 24.84 b	2.08 ± 0.23 a	0.29 ± 0.13 a
	Cu	53.16 ± 3.99 a	50.93 ± 2.04 b	58.33 ± 2.33 c	1.99 ± 0.11 a	0.72 ± 0.14 a
	Zn	33.70 ± 3.71 a	62.50 ± 6.44 b	66.25 ± 5.70 c	1.58 ± 0.23 a	1.42 ± 0.07 a
	Cd/Cu/Zn	50.90 ± 8.38 a	275.00 ± 26.95 a	220.00 ± 17.60 a	1.61 ± 0.26 a	0.18 ± 0.03 a

alone or combined (Tables 2 and S2). By contrast, the gene *AAO*, encoding for the enzyme aldehyde oxidase, catalyzing the final regulatory oxidation of indole-3-acetaldehyde to IAA, was never affected by metal exposure, either in roots or in shoots (Tables 2 and S2).

In the shoots, the gene *IPT*, encoding the isopentenyl transferase, which catalyzes the isopentenylation of AMP in the biosynthetic pattern of CKs (Hedden and Thomas 2006, Miyawaki et al. 2006), was significantly up-regulated after Cd and Cu exposure (from 2.7- to 13.5-fold) ($P \leq 0.001$ for Cu and $P \leq 0.01$ for Cu) (Tables 2 and S2), whereas *CKX*, encoding for the main enzyme involved in CKs degradation pathway (CK oxidase) (Srivastava 2002, Hedden and Thomas 2006) was down-regulated by all the metals, alone or in combination (from 0.1- to 0.3-fold) (Tables 2 and S2). By contrast, the expression of *IPT* and *CKX* in roots was not significantly affected by the metals as compared to the controls ($P \leq 0.05$) (Tables 2 and S2).

In shoots, the expression of *GSH1* and *GSH2* (whose products catalyze the first and second reaction of GSH synthesis, respectively), and of *PCS1* and *PCS2* (the genes encoding for the PCS1 and PCS2 enzymes), were not influenced by metal exposure (Tables 2 and S2). In roots, only GSH1 expression was significantly ($P \leq 0.05$) reduced after exposure to Cd and Zn (Table 2).

Discussion

The exposition of *Arabidopsis* seedlings to the metals determined an effective metal uptake by the roots and an efficient translocation to the shoots (Fig. 1A, B). Taking into account the differences in metal exposure time and concentration employed in other experiments, the levels of Cd and Zn taken up were comparable to those previously found by other authors (Wójcik and Tukiendorf 2004, Brunetti et al. 2011). Regarding the competitive phenomena in root metal uptake, particularly evident for Cd (Fig. 1A), Smeets et al. (2009) observed a significant Cd reduction in the roots of *Arabidopsis* plants simultaneously exposed to $CdSO_4$ (10 μ*M*) and $CuSO_4$ (10 μ*M*) for 24 h, due to an interference of root-shoot transport generated by a phytotoxic effect of Cu in roots. However, the relatively long period of Cd exposure (12 days) determined here an efficient Cd translocation in shoots (Fig. 1B). This high translocation observed is due to the fact that in *Arabidopsis*, at Cd levels up to 50 μ*M*, the symplastic and apoplastic Cd transport through the root cortex and its translocation *via* the xylem are efficient, in comparison to other species (Vangronsveld et al. 2009, Cuypers et al. 2010). The significant concomitant increases in GSH and PCs in roots (Fig. 3A), highlights the importance of GSH/PCs balance in root cells for Cd tolerance and root accumulation, as demonstrated in tobacco (Pomponi et al. 2006). Conversely, in shoots, Cd determined an increase of PCs not accompanied by that of GSH (Fig. 3B), and this unbalance could be the cause of the observed reduction in shoot height, shoot yellowing and rosette diameter, in accordance with the results found in tobacco (Pomponi et al. 2006). It is interesting to point out that, in general, the amount of Cu accumulated in roots appears to be often inversely correlated to the level of Cu tolerance (Colzi et al. 2011; and references therein). Here, *Arabidopsis* seedlings showed a high capacity of Cu uptake and translocation to the shoot (Fig. 1A, B), in contrast to species with high affinity for Cu-rich soils, that usually adopt a Cu-exclusion strategy even at Cu concentrations higher than those used in this work (Faucon et al. 2012). Moreover, in both roots and shoots, Cu exposure induced a low PCs accumulation and did not significantly affect GSH levels (Fig. 3) nor the

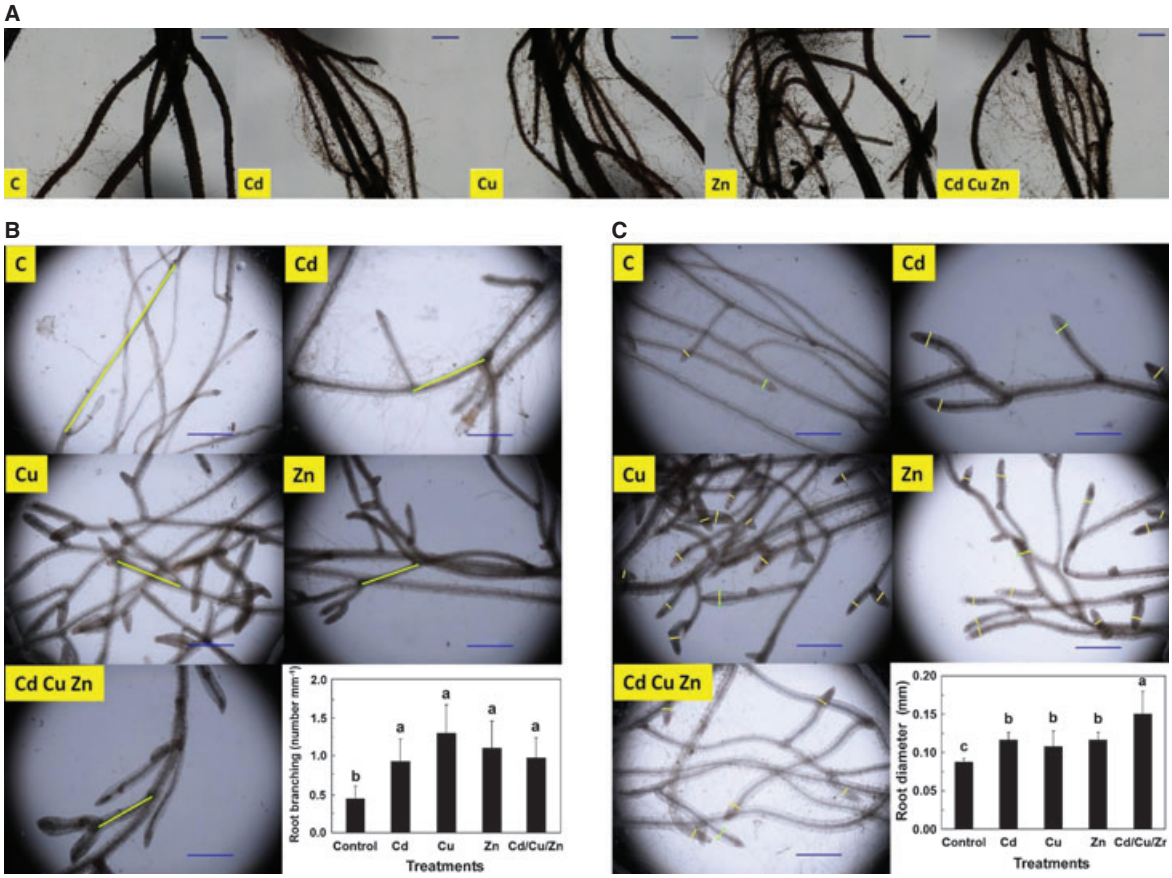

Fig. 2. (A) Root hairs density, (B) root branching, and (C) root mean diameter in *Arabidopsis* seedlings not exposed to metals (Control = C), or exposed for 12 days to 10 μ*M* $CdSO_4$ (Cd), 5 μ*M* $CuSO_4$ (Cu), and 150 μ*M* $ZnSO_4$ (Zn), alone or in triple combination (Cd/Cu/Zn). Measured distances and diameters are highlighted with yellow segments. Scale bars = 0.5 mm. Mean values (n = 10) ± SD with different letters are significantly different between treatments at $P \leq 0.05$, according to Fisher's LSD test.

related gene expressions (Table 2), suggesting that GSH is not directly involved in Cu detoxification and tolerance in *Arabidopsis*, in accordance with Wójcik et al. (2009). Increases in GSH due to Zn supply were present in *Arabidopsis* roots (Fig. 3A), in accordance with results reported in maize and wheat roots (Souza and Rauser 2003, Sanità di Toppi et al. 2009), suggesting a possible GSH-driven Zn translocation into the vacuole, whereas no GSH increase in roots and leaves was reported in bean (Cuypers et al. 2002). Zinc sequestration in roots of *Arabidopsis* is carried out by PCs and particularly by the PC_3 oligomer (this latter was shown to strongly accumulate in the roots of Zn-exposed plants, as in Fig. S2A), possibly contributing to the translocation and compartmentalization of Zn excess into the vacuole, as observed in other species (Lasat et al. 1996, Cuypers et al. 2002). When bound to Cys and thiol-peptide compounds, Zn impairs its ROS production, and the Zn-binding to SH groups can be important for reducing the oxidative damage in plant cells (Cakmak 2000). The three metals given alone accumulated in the roots more than in the shoots, and this trend was maintained when the seedlings were subjected to the triple combination, with Zn showing the lowest translocation to the shoot (Fig. 1B). This result is also consistent with the statement that Zn root–shoot translocation is probably reduced because of a trapping role by root cell walls not influenced by the other metals, according to Roosens et al. (2008).

The metals, above all in combination, modulate auxin and cytokinin levels and their related gene expression

Different approaches have been used in plants to investigate the effects of metals at genomic, transcriptomic and post-transcriptomic levels (Semane et al. 2010). It is known that the activation of responses induced by abiotic stresses, including metals, is mediated through

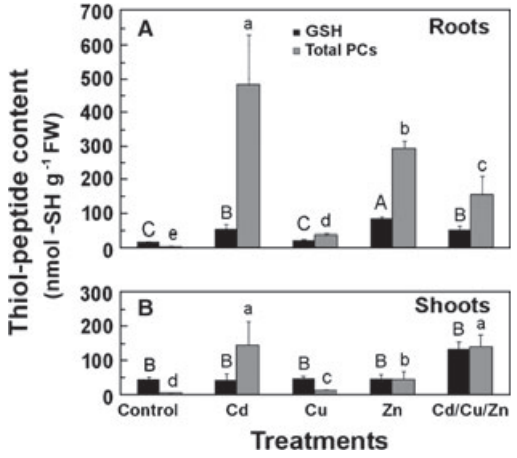

Fig. 3. GSH (black columns) and total PC content (grey columns) in (A) roots and (B) shoots of *Arabidopsis* seedlings not exposed to metals (Control), or exposed for 12 days to 10 μ*M* $CdSO_4$ (Cd), 5 μ*M* $CuSO_4$ (Cu) and 150 μ*M* $ZnSO_4$ (Zn), alone or in triple combination (Cd/Cu/Zn). Mean values (n = 5) ± SD with different letters are significantly different between treatments at $P \leq 0.05$, according to Fisher's LSD test. Capital and lower case letters allow comparisons within GSH and PC levels, respectively.

the synthesis of molecules with signal function, such as phytohormones. The observed changes in IAA levels in the roots of metal-exposed plants, excluding Zn single treatment (Table 1), were likely due to the regulation of *NIT* accompanied by the stable expression levels of *AAO* (Table 2). In particular, the root exposed to the three metals in combination caused the highest *NIT* expression, a stable *AAO* expression (Table 2) and, consequently, the highest IAA level in the root (Table 1). Both these genes did not change their expression levels in the shoots (Table 2), and, in accordance, the IAA levels in shoots of metal-exposed plants were not different from those found in the controls (Table 1).

Trans-zeatin riboside (*t*-ZR) and dihydrozeatin riboside (DHZR) are implied in inhibiting root growth whereas are positively involved in releasing inactive lateral buds, promoting leaf expansion, and delaying senescence (Srivastava 2002). In *Arabidopsis*, *IPT* gene is of basic importance in the regulation of CK biosynthesis, whereas the gene encoding for the main enzyme for CK degradation pathway is the CK oxidase (*CKX*) (Aloni et al. 2006, Miyawaki et al. 2006). In the shoots of *Arabidopsis* seedlings grown in the presence of metals, the significant increase in *IPT* expression and the decrease in *CKX* expression (Table 2) are consistent with the increases of *t*-ZR and DHZR in the shoot (Table 1). Under normal conditions, CK synthesis mainly occurs in the root tip cells and then these phytohormones are transported up to shoot organs (Aloni et al. 2006, Miyawaki et al. 2006). *Arabidopsis* plants were able to ensure shoot CK availability by the up-regulation of *AtIPT* in the shoots under metal stress (Tables 1 and 2). In the shoot, the young leaves are sites of CK synthesis (Nordström et al. 2004). The increase in CKs levels both in the shoot and the root after metal exposures (Table 1) can be interpreted as a detoxification response to counteract the oxidative stress known to be induced by the metals. In fact, elevated levels of CK are advantageous for the survival to toxic heavy metals, as observed by Thomas et al. (2005) in tobacco leaves.

AUXs are known to play a positive role in inducing lateral root formation, and changes in phytohormone homeostasis are involved in reorientation and redistribution of root growth (Dubrovsky et al. 2008). The effect of metals on AUX content and the related alterations in morphogenesis is explored enough (Pasternak et al. 2005, Lequeux et al. 2010), and, moreover, the role of AUXs in root hair formation is well established (Jones et al. 2008). The results here reported show an increase in root hairs density and in root branching consistent with significant increasing levels of IAA in the roots of the seedlings exposed to the metals, alone as in triple combination (Fig. 2A, B). The Cu-induced AUX redistribution is accountable for changing in some morphogenic plant responses, such as root elongation, and formation of root hairs and secondary roots (Pasternak et al. 2005, Lequeux et al. 2010). The exposure to Cd, Cu and Zn in combination, resulting in an over fivefold increasing of IAA level in roots (Table 1), is consistent with the higher level of expression of *NIT* respect to the control (Table 2) and with the notion that also the root tips of the lateral roots are important sites of AUX biosynthesis, e.g. in *Arabidopsis* (Ljung et al. 2005). The observation that the treatments with either Cd or Cu resulted in an increase of IAA level in the roots (Table 1), while the exposure to Zn gives no significant differences in root IAA levels (Table 1), demonstrates that Zn excess does not contribute to the increase of IAA amount obtained with the multiple treatment. This suggests that the strong IAA increase observed in the treatment with the tree metals (Table 1), is essentially caused by Cd and Cu.

It is not surprising that CK increase in triple-exposed plant roots (Table 1) did not affect either growth or branching (Figs 1B and 2B), because the CK level might not be sufficient for contrasting the high amount of IAA, and the related IAA-promotion of root morphogenesis. Conversely, the exposure to single metals and not to the triple combination showed to positively affect GA3 content in roots in comparison with the control (Table 1). In accordance, GA3 is known to counteract the adverse effects of heavy metals (Cd and Pb) on mitotic index

Table 2. Expression levels of the genes involved in phytohormone and thiol-peptide compound metabolism measured by qRT-PCR (controls = 1.000) in roots and shoots of *Arabidopsis* seedlings not exposed to metals (Control), or exposed for 12 days to 10 μ*M* $CdSO_4$ (Cd), 5 μ*M* $CuSO_4$ (Cu), and 150 μ*M* $ZnSO_4$ (Zn), alone or in combination (Cd/Cu/Zn). Mean values (n = 3) ± SD with different letters are significantly different between treatments, according to one-way ANOVA with Tukey post-test ($P \leq 0.05$).

		AtNIT	*AtAAO*	*AtIPT*	*AtCKX*	*AtGSH1*	*AtGSH2*	*AtPCS1*	*AtPCS2*
		(expression level compared to controls)							
Roots	Control	1.000 ± 0.047 c	1.000 ± 0.047 a	1.000 ± 0.233 a	1.000 ± 0.387 ab	1.000 ± 0.116 a	1.000 ± 0.261 a	1.000 ± 0.289 ab	1.000 ± 0.364 a
	Cd	3.403 ± 0.955 a	0.736 ± 0.057 a	0.708 ± 0.207 a	7.635 ± 3.839 a	0.491 ± 0.038 b	0.662 ± 0.113 a	0.883 ± 0.130 b	0.547 ± 0.035 a
	Cu	2.156 ± 0.237 b	1.024 ± 0.143 a	2.211 ± 0.676 a	0.795 ± 0.295 ab	0.691 ± 0.075 a	1.192 ± 0.176 a	1.803 ± 0.130 a	1.412 ± 0.205 a
	Zn	0.453 ± 0.074 c	0.865 ± 0.090 a	0.958 ± 0.073 a	0.432 ± 0.117 b	0.406 ± 0.046 b	0.662 ± 0.082 a	0.732 ± 0.190 b	0.965 ± 0.293 a
	Cd/Cu/Zn	3.537 ± 0.247 a	0.825 ± 0.007 a	2.060 ± 0.198 a	1.010 ± 0.568 ab	0.695 ± 0.013 a	1.204 ± 0.094 a	1.618 ± 0.092 ab	1.217 ± 0.058 a
Shoots	Control	1.000 ± 0.248 a	1.000 ± 0.036 ab	1.000 ± 0.051 b	1.000 ± 0.006 a	1.000 ± 0.139 a	1.000 ± 0.036 a	1.000 ± 0.128 a	1.000 ± 0.308 a
	Cd	1.824 ± 1.026 a	1.734 ± 0.246 a	13.460 ± 4.919 a	0.116 ± 0.012 c	1.224 ± 0.158 a	1.095 ± 0.116 a	1.806 ± 0.172 a	1.093 ± 0.216 a
	Cu	1.032 ± 0.193 a	0.808 ± 0.094 b	6.685 ± 0.724 a	0.177 ± 0.037 bc	1.088 ± 0.191 a	0.984 ± 0.157 a	1.095 ± 0.208 a	0.848 ± 0.094 a
	Zn	0.924 ± 0.176 a	1.009 ± 0.192 ab	5.495 ± 2.027 ab	0.316 ± 0.100 b	1.686 ± 0.516 a	1.380 ± 0.306 a	1.123 ± 0.235 a	1.485 ± 0.386 a
	Cd/Cu/Zn	0.420 ± 0.017 a	1.074 ± 0.102 ab	2.698 ± 1.068 ab	0.099 ± 0.008 c	0.637 ± 0.132 a	0.933 ± 0.173 a	1.925 ± 0.200 a	0.614 ± 0.292 a

and some metabolic mechanisms, e.g. in bean plants (Mansour and Kamel 2005). No effect of the single and the triple metal combination on ABA levels was observed both in roots and shoots (Table 1), highlighting that AUXs and CKs were the hormones specifically affected by the metals.

Taken together, these results point to a significant influence of the three metals on *Arabidopsis* hormonal status (Table S2); consequently, a combination of Cu, Cd and Zn seems to act synergically in inducing an univocal metal-pollution response.

The combined presence of the metals affect root morphogenesis

Even though the key components of the cell cycle and signal-transduction pathways that promote and attenuate AUX-dependent lateral root initiation and elongation in *Arabidopsis* have been identified (Casimiro et al. 2003, Peleg and Blumwald 2011), there are no mechanistic studies in this species on the relationships between Cd/Cu/Zn-induced changes in phytohormones and the related modifications in root morphology. Pasternak et al. (2005) observed that in *Arabidopsis* Cu-exposed plants (30–100 μ*M* $CuSO_4$), root hairs density was significantly increased and an acceleration of the emergence of lateral roots occurred, with a consequent increase in the number of root tips. In carrot plants grown in vitro, a prolonged exposure to Cd (2–7 days) anticipated and stimulated the production of lateral root primordia, as well as the development of primary and secondary xylem (Sanità di Toppi et al. 2012). Thus, the increase in root branching and in the root diameter observed (Fig. 2B) could be imputed to the alteration that metals trigger to the hormonal balance, in particular to the AUX/CK ratio (Pasternak et al. 2005, Marschner 2012). The increased root hairs density (Fig. 2A), lateral root formation (Fig. 2B) and increase in mean root diameter (Fig. 3C) could be functionally related to stress avoidance mechanisms. In fact, the lateral roots with their hairs could allow the Cd/Cu/Zn-stressed root system to maximize the rhizosphere exploration, in order to attempt the recruiting of as many free-metal-patches in the culture medium (and in the soil) as possible. Elevated root system plasticity is one fundamental mechanism driving the response to the variation of mineral availability in the soil (Marschner 2012). Therefore, the remodeling of the root architecture in response to metals (e.g. Cd) has been proposed as a pollution 'escaping' strategy (Sanità di Toppi et al. 2012). In this view, the observed increase in root diameter (Fig. 3C) in metal-exposed plants could be a consequence of metal-induced xylogenesis (Schützendübel et al. 2001, Ďurčeková et al. 2007), that acts as a barrier to protect root from the metals. The root thickening was likely promoted by CKs accumulation in *Arabidopsis* roots (Table 1), that causes rhizodermal and parenchymal cell proliferation (Srivastava 2002, Hedden and Thomas 2006).

The morphological changes induced by the metals also reflect the different levels of GA3, a hormone involved in the promotion of elongation in axial organs, such as main root (Hedden and Thomas 2006). The root level of GA3 in Cd/Cu/Zn-treated plants was similar to that of control seedlings (Table 1); however, the roots were shorter (Fig. 1C), suggesting that the AUXs/CKs unbalance [IAA/(*t*-Zr + DHZR) = 29.6 in CD/Cu/Zn roots vs 9.8 of controls] had negatively affected a possible over-production of GA3 aimed to compensate the root length reduction induced by the metals. The AUXs/CKs ratio is determinant for governing many growth aspects, e.g. the axillary bud outgrowth, through a control on

GA3 and ABA levels (Shimizu-Sato and Mori 2001). This latter is known to act as a general inhibitor of root and shoot growth and metabolism, even though these effects vary with tissue and developmental stage (Srivastava 2002). Generally, ABA accumulates in response to numerous stresses, such as drought, high salt and cold, where an overlap in the expression pattern of stress genes occurs (Tuteja 2007), but not in response to metal treatments (Srivastava 2002, Hedden and Thomas 2006). Indeed, the results show no significant differences in ABA levels between control and metal-exposed seedlings in both roots and shoots (Table 1), indicating that ABA is not involved in the growth inhibition because of the stress induced by the metals, and its level is not affected by the metal-caused alteration in the AUXs/CKs ratio.

Conclusions

Our study demonstrated that the molecular, biochemical, physiological and morphological characteristics of *A. thaliana* seedlings are markedly affected by the exposure to Cd, Cu and Zn. From the analysis of the results, it appeared that morphological root changes could be part of an integrated hormonal response against the metal stressors, mainly governed by the auxin/cytokinin ratio.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used for the amplification of gene sequences.

Table S2. Correlation coefficients (r values) for the relationships among metal treatments (Cd, Cu, Zn and Cd/Cu/Zn) and the different root physiological and biochemical properties in roots and shoots of Arabidopsis seedlings.

Fig. S1. Biplot of the principal component analysis of roots and shoots of Arabidopsis seedlings not exposed to metal, or exposed for 12 days to10 μ*M* $CdSO_4$, 5 μ*M* $CuSO_4$, and 150 μ*M* $ZnSO_4$, alone or in combination.

Fig. S2. GSH and PC oligomer content in roots and shoots of Arabidopsis seedlings not exposed to metal, or exposed for 12 days to 10 μ*M* $CdSO_4$, 5 μ*M* $CuSO_4$, and 150 μ*M* $ZnSO_4$, alone or in combination.

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