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<AT>Screening Arabidopsis mutants in genes useful for phytoremediation

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Research area: Biological Processes for HM Removal

<ABS-Head><ABS-HEAD>Graphical abstract
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<ABS-HEAD>Highlights► A screening of 7,000 mutants in a medium containing three heavy metals was done ► Protein metabolism and signalling mutants may regulate plant response to metals ► Metal transport and localization in plants may be crucial for phytoremediation

<ABS-HEAD>ABSTRACT

<ABS-P>Emissions of heavy metals have risen over the past 200 years and significantly exceed those from natural sources. Phytoremediation strategies may be able to recover soil productivity in self-sustaining ecosystems; however, our knowledge of the molecular mechanisms involved in plant heavy-metal perception and signalling is scarce. The aim of this study was to assemble a ``molecular tool box" of genes useful for phytoremediation. To identify mutants with different heavy-metal-tolerance, we first selected a medium from mixtures containing three metals based on their presence in two Spanish mining areas and then screened about 7,000 lines of Arabidopsis T-DNA mutants and found 74 lines more resistant and 56 more susceptible than the wild type (WT). Classification of the genes showed that they were mainly linked to transport, protein modification and signalling, with RNA metabolism being the most representative category in the resistant phenotypes and protein metabolism in the sensitive ones. We have characterized one resistant mutant, *Athpp9* and one sensitive, *Atala4*. These mutants showed differences in growth and metal translocation. Additionally, we found that these mutants keep their phenotype in amended former soils, suggesting that these genes may be useful for phytoremediation and the recovery of contaminated soils.

<KWD>Abbreviations: Cd: cadmium; MDA: malondialdehyde; TBA: 2-thiobarbituric acid.

<KWD>Keywords: Arabidopsis; ATPase; copper; cadmium; chaperons; chrome; heavy metals; mining; oxidative stress; screening

INTRODUCTION

Heavy metals are characterized by a density of ≥5,0 g cm^{-3} and some of them are in the 10 most hazardous contaminants of U.S. Environmental Protection list [1]. Some of these metals, such as copper (Cu) or iron (Fe) are necessary for a proper plant growth at low concentrations but become toxic at high concentrations [2]. The accumulation of trace elements, especially heavy metals, in soil produces a reduction in the soil quality and it is toxic for plants, giving rise to a loss of vegetation cover and land degradation [3]. Soil degradation is also a major source of pollution of the surrounding environment, by wind dispersion and water erosion processes [4,5]. Mining areas are an example of heavy-metal accumulation and the abandonment of these areas has a negative influence on economic and environmental development [6] as contamination not only involves soils but also air, aquifers and the food chain [7,8]. Recovering contaminated soils with revegetation is a good strategy to cope with this health and environmental problem as it is a relatively low cost approach [9]. Additionally, plant cover may reduce wind dispersion and roots may prevent water erosion and accumulate part of the heavy metals from the soil [10–12]. To use plants more efficiently for phytoremediation of contaminated soils, it is helpful to know how plants take up and metabolize heavy metals and identify the rate-limiting steps involved [13]. Our current knowledge about the metabolism of heavy metals in plants and genes associated with this is rather limited and the genes responsible for tolerance or hyper-accumulation have not yet been well established [13]. Different transport-related genes have been cloned and functionally characterized in Arabidopsis and other plant species. Particularly important are HMA, ZIP, NAS, MTP, NRAMP and YSL gene families that have a significant role in metal uptake, compartmentalization and transport [14]. However, the cellular response to metal accumulation uses different signalling mechanisms and is very complex [15] especially related to a cross-homeostasis between different metals [16]. Most of the studies however, have analysed the effect on the plant of just one specific heavy metal and it does not represent actual field conditions.

The aim of this study was to obtain a hub of genes related with heavy-metal tolerance. Studies on the soils from two abandoned mining areas were used to establish a heavy-metal mixture in order to screen around 7,000 T-DNA insertion lines and look for genes related with heavy-metal tolerance. Thus, heavy metal concentrations and pH from two contrasting mine soils from the south of Spain (Andalusia) were used to select the media: 1) located in río Tinto (Nerva-Huelva, western Andalusia) one of the largest sulphide deposits in the world, with extremely acid pH and heavily contaminated with toxic elements and 2) located in Alquife (Granada, eastern Andalusia) from an abandoned iron ore mine, with a basic pH and slightly polluted [6]. Finally, a medium containing Cd, Cr and Cu was selected to do the screening and 120 lines with a differential tolerance regarding the wild type (WT) were found and classified. Two of these lines were further characterized and their response in a soil extract from Riotinto

with two different amendments was analysed to check the validity of the screening and the possible use of genes selected for phytoremediation.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (Col-0) seeds, WT and 6,760 T-DNA mutants from the SALK Institute, provided by the European Arabidopsis Stock Centre (NASC, ID: N27941), were surface sterilized with ethanol 70% (v/v) for 2 min and after rinsed three times in autoclaved distilled water, and finally placed in distilled water at 4ºC for 2 days. For the screening, Arabidopsis plants were grown vertically in square Petri dishes (10x10 cm) filled with Hoagland medium at 22/20ºC under long day conditions, a photon flux density of 100 µE and 60-65% relative humidity in the growth chamber. Plates were scanned at the indicated times (7 and 12 days) to analyse root length.

Selected plants were checked for homozygosis of T-DNA gene insertion by PCR analysis of genomic DNA using primers cited in Suppl. Table S1.

Culture medium and treatments

We analysed Riotinto soil, a mining area (37º 42' 4.5'' N 6º 33' 35.1'' W) located in the Iberian Pyrite Belt, and found the following concentration of some potential hazardous metals, such as Pb (235 µM), Cu (196 µM) and Cd (50 µM). It is a very acid soil with low organic carbon (OC) content and high electrical conductivity (EC), [17]. Alquife soil is a mine dump sited in Alquife (Granada, Spain) located SE of the Iberian Peninsula (37º 11' 50'' N 3º 6' 4'' W). This soil exhibits an alkaline pH with low EC and OC contents [17] and we found a locally hazardous concentration of Cr (158 µM), Cu (92 µM), Ni (74 µM) and Cd (50 µM). Initially, to select the final medium for the screening, three different mixtures each containing three heavy metals were used. The initial concentration of metals was selected on the basis of the heavy-metal concentration in the former soils to allow plant growth: 1) RT1: containing Cd (50µM), Cu (50µM) and Pb (160µM) at pH 5.0; 2) AL1: containing Cd (50µM), Cu (100µM) and Cr (150µM) at pH 8.0 and 3) AL2: containing Cd (50µM), Cu (100µM) and Ni (75µM) at pH 8.0. Thus, seeds were sown on plates filled with half-strength Hoagland solution [18] containing sucrose (30 g/l) and phytoagar (8 g/l).

When required, plants were grown in hydroponic culture medium with half-strength Hoagland nutrient solution [18], for 4 weeks and then the medium was supplemented (HM mix) or not (control) with the final concentration selected: Cu, Cd and Cr (50, 50 and 100 µM, respectively).

For the experiments with amended soils, the soil from an abandoned mine in the Riotinto mining area was used. Amendment 1 was produced by composting an urban sewage sludge (SVC) and agricultural by-products (50:50, w:w) and amendment 2 was produced from bottom ashes (BA), a by-product of biomass combustion provided by a local biomass power plant using as fuel a mixture of biomass composed by energy crops and olive-mill waste (40:60, w:w) [15]. Milli-Q water extracts of unamended and amended soil were prepared (1:10 w:v) by shaking (8 h) and centrifuging (3500 rpm for 10 min) to remove particulate matter. Water extracts of unamended and amended soil were used for plant growth [15]. An optimum combination of soil and amendments for vegetation growth was selected according to Sevilla-Perea et al. [19].

Metal and macronutrient content

At the time of harvesting, the plants grown in hydroponics were divided into roots and leaves. The roots were rinsed twice with double-distilled cold water to eliminate extra metal attached to the surface and the roots and leaves were then oven-dried at 60ºC for 72 h. Next, 50 mg of dry tissue was ground and digested with 5 ml of concentrated HNO_3, using an open digestion system as describe elsewhere [20]. The concentration of Cd, Cu, Cr, micronutrients and macro-elements were determined by inductively coupled plasma-atomic emission spectrometry (ICP-MS). The translocation factor was determined as the ratio of metals content in shoots related to the roots (S/R)*100, similar to that described by Zhang et al. [21].

Gene expression

Total RNA was isolated from Arabidopsis plants by Trizol reagent (Invitrogen) according to the manufacturer's protocol. 1 µg RNA was used as a template for the reverse transcriptase (RT) reaction (iScriptTM cDNA Synthesis, Bio-Rad). Quantitative real-time PCR was performed on an iCycler iQ5 (Bio-Rad) as described elsewhere [22]. Amplification efficiency was calculated using the formula ($E = [10(1/ a) - 1] \times 100$) where ``a'' is the slope of the standard curve. The relative expression of each gene was normalized to that of *TUB-4* and the analysis of the results was done using the comparative critical threshold (ΔΔCT) method [23].

Analysis of data

<PA>Primary root elongation was determined by Image Tool 3.0 software. MapMan software was used to classify the genes of mutants with a more sensitive/resistant phenotype (http://mapman.gabipd.org/; [24]). All treatments included at least three biological replicates. Data processing and statistical analyses (pair-wise comparisons using t-tests) were carried out using Excel (Microsoft Excel 2010). Error bars representing standard deviation (SD) are shown in the figures and the data presented are means of at least three independent experiments. The significance level is represented in the figures by asterisks ($P < 0.05$: *; $P < 0.01$: **; $P < 0.001$: ***). **RESULTS Medium selection from different metal concentrations**

To identify mutants with different sensitivity to heavy metals, we first selected the composition and concentration of the heavy metals in the medium based on the concentrations present in the original soils from Riotinto and Alquife [6]. We selected different heavy-metal mixtures (HM mix) each containing three heavy metals as described in the material and methods section. Arabidopsis WT seeds did not germinate with the concentration of heavy metals present in the soils (data not shown) so the original concentration of heavy metals was reduced gradually to obtain the maximum concentration suitable for screening that allowed the morphological analysis of seedlings, basically root growth (Fig. 1). As expected, all the mixtures analysed arrested root growth, this effect being more pronounced with the time of exposure (Fig. 1B). HM mix selected for further studies was AL1, containing Cd (50 µM), Cu (50 µM) and Cr (100 µM), because they produced intermediate toxicity symptoms in plants and the concentration of heavy metals was as close as possible to the former soil (Fig. 1 A, B) whilst plants hardly grow in RT1 and AL2 conditions.

Screening for differential metal tolerance and identification and classification of selected mutants

Under the medium selected, we screened a one-allele set of 6,867 confirmed T-DNA SALK insertion lines. Each line (L_n) representing an insertion in a different locus. We

used four seeds per line for an initial screening obtaining 164 more resistant and 74 more sensitive lines than the WT plants (Fig. 2A). We further confirmed the sensitivity of the lines by a second analysis in the same conditions, by using at least 30 plants per line. In this way we validated a more resistant phenotype for 74 lines and a more sensitive phenotype for 56 lines (Fig. 2B). We also checked the phenotype of these mutant lines compared to WT in control conditions and found that 10 lines of the sensitive ones were more sensitive. Thus, we selected the 46 sensitive lines that had no differences in growth under normal conditions for further analysis.
Genes affected by T-DNA insertion were identified for each of these mutants (Suppl. Table S2 and S3) and classified by Mapman [24]; (Fig. 3 A and B). We found genes related not only to transport but related to gene regulation, protein modification, stress and signalling (Fig. 3). The main category in sensitive mutants is protein metabolism (about 25%), with the second one being mutants related with RNA metabolism (11%). The latter however, was the most represented in the resistant mutants (12%), followed by DNA and protein metabolism with 10% each. It is interesting to note that stress related genes have 7% occurrence in sensitive mutants, whilst just 1% occur in the resistant ones. On the contrary, the transport category is more represented in resistant mutants (7%) than in the sensitive ones (4%).

HPP9 and ALA-4 are involved in resistance to heavy metals

We selected one resistant and one sensitive line related with transport to obtain deeper insight into their possible role in the response to heavy-metal stress and their use for phytoremediation. The resistant line was *Athpp9* with the T-DNA insertion in the *HPP9* gene, a metallochaperone-like protein [25], and the sensitive one was *Atala4* with the T-DNA insertion in the *ALA4* gene, belonging to the P-type ATPase ion pumps family [26]. Neither of the genes has been characterized so far. We first established the homozygosis of both lines (Suppl. Fig. S1) and confirmed just one T-DNA insertion back-crossing the mutants with WT plants. Then we confirmed the phenotype of these lines in response to the HMmix, with *Athpp9* showing a longer root and *Atala4* a shorter root compared to the WT seedlings after 7 days' growth (Fig. 4A and B). No differences were found in germination rate, only *Atala4* had a slightly lower rate in control and HMmix conditions (Suppl. Fig. S2). Additionally, we observed that the *ALA4* gene was induced in WT seedlings after 7 days' growth in the HMmix compared to the control medium, whilst no statistical differences were found for the *HPP9* gene under these conditions (Fig. 4C).

Heavy-metal uptake and translocation are affected in *Athpp9* and *Atala4* mutants

To obtain enough material for the ICP analysis, WT plants and *Athpp9* and *Atala4* mutants were grown in hydroponic conditions for three weeks and then the HM mix was added for 24 h. A higher accumulation of heavy metals (Cd, Cr and Cu) was observed in the roots of both mutants compared to the WT plants and the translocation to the shoots was also altered (Fig. 5).
Thus, metal translocation in *Atala4* was higher than in WT by 2.1, 2.0 and 1.4 times for Cd, Cr and Cu, respectively (Table 1), whilst in *Athpp9* mutant, translocation was reduced by 2.5, 2.1 and 3.6 times for Cd, Cu and Cr, respectively in comparison with WT plants (Table 1). Thus, this factor may be important in the observed phenotype of these mutants.
Interestingly, Fe translocation in *Athpp9* was 10.9 times higher than in the WT plants under control conditions; however it was 23 times lower than the WT plants in HM mix conditions (Suppl. Table S4). Mn and Zn translocation was lower than in WT, even

under control conditions, but the uptake under control conditions was 2 and 3 times higher than in WT plants, respectively (Suppl. Table S4). Additionally, the *Athpp9* mutant accumulated macronutrients in roots under control conditions, especially with an increase in Ca, K, S and P content of about 1.5, 2.5, 2.7 and 4.0 times compared to WT content (Suppl. Table S5). On the contrary, Mg concentration was almost one third of the content observed in the WT plants. Mg translocation under stress conditions was also affected in *Athpp9* mutant (Suppl. Table S5). *Athpp9* also accumulated more K, S and P in roots, under HMmix stress (Suppl. Table S5). No differences were found in roots for the *Atala4* mutant; just an increase in P content under control conditions and the translocation to the leaves was almost three times higher than in the WT plants (Suppl. Table S5).

The analysis of *HPP9* expression showed very early induction in roots growing in hydroponics under HM mix (Fig. 6A), whilst no expression in leaves was observed (data not shown). Thus, *HPP9* was significantly induced after 30 mins' treatment, but it was repressed after 24 and 48 h (Fig. 6A). *ALA4* was induced, however, after a long-time HM mix treatment (24 and 48 h) in leaves (Fig. 6B) and no significant differences in *ALA4* expression were found in roots (data not shown).

***Athpp9* and *Atala4* response to amended soils**

The toxic conditions of the original soil (extreme pH, high electrical conductivity and hazardous element toxicity) together with physical conditions such as compaction, high apparent density and cementation strongly constrain the establishment of vegetation and amendment of soils is recommended [17,27]. So, in order to validate screening results we analysed growth and stress parameters in *Athpp9* and *Atala4* mutants in mine-dump soil extracts from Riotinto with two different amendments using: 1) compost or 2) bottom ashes. Germination rate was earlier in both mutants compared to the WT and the differences were significant for *Athpp9* for both amendments used (Fig. 7 A and C). Root length was also greater in *Athpp9* mutants and the difference was significant for amendment 1 (Fig. 7 E), whilst the *Atala4* mutant root growth was significantly less for both amendments used (Fig. 7 B and D). Therefore, both mutants conserved the phenotype observed in the screening (Fig. 7E). Interestingly, both mutants had less lipid peroxidation used as a marker of stress, after 5 days' growth in soil plus amendment 1 (Fig. 7 F).

DISCUSSION

Phytoremediation is an important tool for removing heavy metals and other contaminants from polluted soils [9] and improvement in this field depends on an in-depth scrutiny of the genes involved in plant response mechanisms to heavy metals [14]. Regulation of different described genes involved in heavy-metal tolerance appears to be crucial in mirroring hyper-accumulation in high-biomass species useful for phytoremediation [14]. Usually, analysis of genes involved in metal tolerance and accumulation have been made with a single gene and with a single metal, but for phytoremediation probably a greater insight into tolerance to a HMmix similar to those occurring in contaminated soil and a multi-gene strategy is needed. In this study, we have selected a medium containing three heavy metals based on the concentrations and conditions present in two Spanish soils from abandoned mines, Riotinto and Alquife. After the selection of the medium a screening with 6,867 confirmed T-DNA SALK insertion lines was done, obtaining 120 mutants with a differential sensitivity to the medium than the WT. Functional categorization of these lines identified members of different protein families including protein metabolism, redox-and stress-related

proteins, signalling-regulating proteins, etc. suggesting that resistance to metal stress involves practically all the physiological processes in the plants as small-scale experiments and whole-genome approaches have shown in recent years [28–30].

Protein metabolism and signalling in plant tolerance to heavy metals

Besides transcriptional control, different post-transcriptional mechanisms (PTM) regulate plant responses to heavy metal stress [31]. Actually, one of the major groups found after the selection of mutants, especially in the sensitive ones, is related with protein metabolism, mainly devoted to post-translational modifications involving kinases and phosphatases and to degradation via ubiquitin involving the E3-SCF-FBOX pathway. Phosphorylation/dephosphorylation processes have been involved in plant response to heavy-metal stress in different plant species and tissues [28,29,32,33] showing that this PTM is important in signalling events after metal treatment leading to the survival of the plant or not. Additionally, at least four of the mutants selected showed disturbances in proteins related to E3 ubiquitin ligases, an activity that provides specificity during ubiquitination and in many cases promotes degradation of substrates at the 26S proteasome. This activity appears to regulate plant signalling and plant adaptation to stress [34]. Moreover, signalling-regulating proteins including transcription factors (TFs) from different families have been shown to be induced by heavy-metal stress [28,29,33,35,36] and we have found 14 mutants with a T-DNA insertion in a TF with a different sensitivity compared to the WT plants. Especially important is the MYB family in the resistant mutants selected as a third of these mutants belong to this family.

Transport-related proteins contribution to metal tolerance: HPP9 and ALA4

An important topic in the heavy-metal effect and plant response is related to transporters, as heavy metals use these proteins to enter the plant. Plant cells contain a large number of transporters and these proteins must be expressed in the appropriate amount and cell type [37]. In addition, it has been shown that depending on environmental conditions, intracellular distribution of some transporters is highly regulated, not only at transcriptional level but also by post-translational regulatory mechanisms in order to maintain nutrient homeostasis [37]. In this screening, 10% of the mutants found were related with transport. We selected two of these transport-related proteins to get a deeper insight into the plant response to heavy-metal stress, a metallochaperone-like protein and a P-type ATPase ion pump [25,26]. In plants, metallochaperones have diversified into a large family including two types of proteins: heavy-metal associated plant proteins (HPPs) and heavy-metal associated isoprenylated plant proteins (HIPPs). In Arabidopsis, 22 HPPs have been identified, including AtATX1 and AtCCH with a predominant function in Cu delivery to P-type ATPases and trafficking, respectively [38,39]. Little is known about the other components of the family including HPP9. Our results show induction of *HPP9* in response to HMmix in roots, suggesting a role at the very early plant response to the stress. Additionally, this metallochaperone may be involved in metal translocation from roots to leaves as this is impaired in *Athpp9* mutants, with Fe translocation being mainly affected under HM mix stress. Translocation of macronutrients is also affected in this mutant, whilst P translocation is reduced and Mg is increased. Further analyses are necessary however, to determine specificity of the HPP9 protein. The reduction of heavy-metal translocation in *Athpp9* mutants compared to the WT plants may be one of the reasons for the increased resistance to the stress as has been shown for some hyperaccumulators [40].

On the other hand, ALA4 is a P-type ATPase ion pump, belonging to a family of 45 genes in Arabidopsis; divided into five sub-families depending on the ions they transport [41]. ALA4 belongs to P_4 sub-family and only ALA1 has been characterized [42], showing that this gene is involved in cold tolerance of Arabidopsis plants. ALA1 [42] and yeast DRS2 [42,43] have been implicated in flipping amino-phospholipids. Nevertheless,none of the transport capabilities of these pumps have been characterized and it is interesting that a phenotype of *drs2* cells is sensitive to Zn^{2+} and Co^{2+} [44]. In this study, we have found that the *Atala4* mutant is more sensitive than the WT plants to heavy-metal stress and that there is an induction of the gene after 24 h of HM mix treatment, which was significant only in the leaves. Although it has been proposed that ALA4 may be involved in the transport of phospholipids [41], our results suggest that this protein may be involved directly or indirectly, in heavy-metal transport or translocation. In fact, *Atala4* mutants accumulate more heavy metals than the WT, not only in the roots but mainly in the leaves with an increased translocation from roots to shoots. Similar results have been found for Fe translocation under HM mix stress while the contrary takes place with macronutrients, except Mg. *Atala4* mutants are also more sensitive to soil extracts from the Riotinto mine, regardless of the amendment used, showing that this protein is somehow important for resistance in soils contaminated with heavy metals.

The application of amendments to the soil is necessary to improve its conditions, leading to an increase in the germination and growth of the plants as seeds rarely germinate in the original soil [19]. We analysed growth and stress markers in plants growing in amended soil extracts to check the validity of our screening and to analyse the possible use of the selected mutants in real soils. Thus, the soil extract was amended with compost of urban sewage sludge (SVC, amendment 1) and bottom ashes from biomass combustion (BA, amendment 2) which are known to affect the pH, electrical conductivity (EC) and toxic element extractability of the soil. It is important to note that both mutants analysed not only conserved the resistant/sensitive phenotype obtained in the screening, but also improved their growth in the presence of amendments, thus supporting the beneficial effects of amendments of the soil for plant growth.

Concluding remarks and perspectives

In this study, we have found 120 genes involving different categories, which may have a function in plant resistance to heavy metals. Two of the mutants characterized in more depth and related with transport, showed that the translocation of heavy metals to the leaves appears to be crucial in their phenotype and may be useful for phytoremediation techniques. Future and more specific studies should be done to establish the contribution of each gene selected in this screening in the resistance process to heavy metals in order to improve phytoremediation techniques by using the appropriate plants.

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None of the authors have any conflict of interest to declare.

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<Figure>**Figure 1:** Arabidopsis (Col-0) root length growth after 7 (A) and 12 d (B) in different heavy metal mixtures (HM mix; Cd:Cu:Cr; µM). The results are means of 3 independent experiments ± SE.

<Figure>**Figure 2**: Representative image of a plate with 12 lines (L_n) growing in the medium selected (A) for screening. We used four seeds per line for an initial screening and further confirm a more resistant phenotype for 74 lines and a more sensitive phenotype for 56 lines in a second analysis with at least 30 plants per line (B).

<Figure>**Figure 3:** Pie chart shows the MapMan classification in different categories, and their percentages, according to the functions of the genes affected by the T-DNA insertion in the resistant (A) and sensitive (B) mutants selected.

<Figure>**Figure 4**: Phenotype (A) and root length after 7 d of growth on the HM mix (B) of WT, *Athpp9* and *Atala4* mutants; and the relative expression of *ALA4* and *HPP9* genes in WT seedlings after 7 days of growth in the HM mix compared to control medium. Data represent the mean ± standard error (error bars) of at least three independent experiments, each with three replicates. Asterisks denote statistically significant differences ($P < 0.05$: *; $P < 0.01$: **; $P < 0.001$: ***) according to T-test.

<Figure>**Figure 5**: ICP analysis for Cd, Cr and Cu determination in WT, *Athpp9* and *Atala4* mutants in shoots and roots. Mutants were grown in hydroponic conditions for three weeks and then the HM mix was added for 24 h. Data represent the mean ± standard error (error bars) of two independent experiments, each with three replicates. Asterisks denote statistically significant differences ($P < 0.05$: *; $P < 0.01$: **; $P < 0.001$: ***) according to T-test.

<Figure>**Figure 6**: Analysis of relative expression by qRT-PCR of *HPP9* (A) and *ALA4* (B) genes in WT roots and shoots growing in hydroponics under HM mix treatment. Data represent the mean ± standard error (error bars) of at least three independent experiments, each with three replicates. Asterisks denote statistically significant differences ($P < 0.05$: *; $P < 0.01$: **; $P < 0.001$: ***) according to T-test.

<Figure>**Figure 7**: Germination and root length of WT and *Athpp9* and *Atala4* mutants growing in Riotinto mine soil extract amended with compost (A, B, E) and bottom ashes (C, D).Lipid peroxidation measured as malondialdehyde (MDA) content of WT, *Athpp9* and *Atala4* mutants after five days of growth in compost amendment 1 (F). Data represent the mean ± standard error (error bars) of at least

three independent experiments, each with three replicates. Asterisks denote statistically significant differences ($P < 0.05$: *; $P < 0.01$: **; $P < 0.001$: ***) according to T-test.

<Table>Table 1: Cadmium, copper and chrome translocation from roots to shoots of 21-day-old *Arabidopsis thaliana* plants exposed for 1 d to Cd 50µM, Cu 50 µM and Cr 100 µM.

	WT	***Athpp9***	***Atala4***
Cd(mg/Kg)	1.61±0.20[a]	0.63±0.09[b]	3.32±0.23[c]
Cr(mg/Kg)	1.63±0.28[a]	0.77±0.10[b]	3.28±0.31[c]
Cu(mg/Kg)	2.44±0.32[a]	0.68±0.11[b]	3.47±0.12[c]

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