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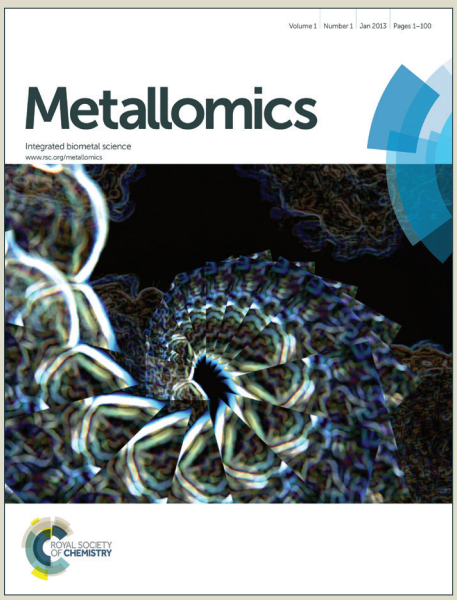

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ARTICLE

Modulation of Zn/Cd P_{1B2}-ATPases activities in Arabidopsis impacts differently on shoot and seeds Zn and Cd contents

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Zn is an essential microelement for all living cells and Zn deficiency is widespread in world's population. At the same time high Zn concentration and low Cd concentration are environmental toxics. Both Zn and Cd are transported *in planta* via Zn/Cd HMA transporters. Engineering of HMAs expression in plants may provide a way for Zn biofortification of food as well as phytoremediation of polluted soils. In the present study we assessed the impact of Zn/Cd HMAs invalidation/overexpression in *Arabidopsis thaliana* on Zn and Cd translocation from the roots to the shoot and in Zn grain filling. Overexpression of *AtHMA4* had a large impact on Zn and Cd translocation and resulted in a 3-fold higher potential of Cd and Zn extraction from an industrial soil highly contaminated by Zn, Pb and Cd. Despite *AtHMA4* overexpressing lines presented a higher Zn concentration in the shoot, the Zn content in seeds was found lower than in wild type plants. Our results indicate that *AtHMA4* overexpression is an efficient tool to increase the root to shoot translocation of Zn and Cd in plants. Concerning biofortification of seeds this study underlines the needs of specific promoters to drive an expression pattern of the transporters in favour of Zn grain filling.

Introduction

Zinc is an essential micronutrient for the embryonic development, growth and reproduction of all organisms. It plays a major role as a cofactor and a structural element in macromolecules and is required for the activity of an estimate of 300 proteins including transcription factors regulating gene expression and enzymes involved in metabolic processes and ROS detoxification[1,2]. In Human it plays critical roles in the nervous, reproductive and immune systems. Over 25 to 30% of the world's population is affected by Zn deficiency according to Maret & Sandstead[3] and the World Health Organization (http://www.who.int/publications/cra/chapters/cra/chapters/volume1/0257-0280.pdf). Such deficiency is mostly encountered in poor or recently developing countries and results from calcareous or alkaline soils with a low Zn bioavailability but also from economical limitations to fertilization and a lack of dietary diversification. Unfortunately, staple foods derived from cereals such as rice and wheat, feeding at least fifty per cent of the world population, are generally poorer in Zn content than other plant seeds[4]. Use of Zn fertilisers poses an economical problem and on the long-term questions the reserves of Zn estimated at the current rate of consumption to be exhausted within 60 yrs[5].

Compared to recursive needs in Zn fertilization of soils, improving crops to raise food Zn content in the edible parts would be an elegant and cost-effective solution[6,7]. Recent advances in the understanding of molecular processes associated to Zn nutrition in plants open the way to genetically increase Zn content in crops. Zn nutrition in plants associates complex mechanisms involving many transporter families including ABC, CDF, ZIP, P-ATPase and Nramp together with various organic and peptidic chelators[6,8,9]. All these actors participate to the acquisition, allocation, compartmentalization and homeostasis of Zn *in planta*. Following the pioneering work of Hussain and *col.*[10], two members of the P_{1B2}-ATPase heavy metal-transporting P-type ATPase subfamily, namely AtHMA2 and AtHMA4, have been recognized as the major transporters involved in the root to shoot translocation of Zn in *Arabidopsis thaliana*[10]. No visible growth phenotype was associated with soil-grown *Athma2* or *Athma4* single mutants[10], despite *Athma4* mutant had lower shoot Zn content than wild type plants. In contrast, double mutants *Athma2*

Athma4 were dwarf, sterile and Zn deficient in the upper parts. All these symptoms were rescued by feeding *Athma2 Athma4* mutants with a high Zn concentration in the nutrient solution pointing to a major role of both AtHMA2 and AtHMA4 in Zn root to shoot translocation.

These and following studies[11,12] confirmed an essential role for these transporters in the translocation of Zn but also Cd, a strongly toxic heavy metal. This is in accordance with the observation that *35S::AtHMA4 A. thaliana* overexpressing lines accumulated higher levels of Zn and Cd in leaves[13]. Moreover, their implication was elegantly demonstrated in Zn/Cd hyperaccumulator species such as *Arabidopsis halleri* which naturally accumulates and tolerates leaf concentrations as high as 2.2% zinc and 0.28% cadmium in dry biomass[14]. Zn hyperaccumulation and full hypertolerance to Cd and Zn in *A. halleri* were found dependent on the metal pump AtHMA4 through a combination of modified cis-regulatory sequences and copy number expansion, 3 orthologues of *AtHMA4* being found in *A. halleri.* Four copies of *AtHMA4* orthologues have recently been identified in another Zn/Cd hyperaccumulator *Noccaea caerulescens* and their expression levels determine the capacity to tolerate and accumulate cadmium in different ecotypes[15].

AtHMA3, a third member of the P_{1B2}-ATPase subfamily in *A. thaliana* has been characterized through heterologous expression in yeast mutants and reverse genetics and found to participate in Zn and Cd internalisation into the vacuole[16]. Interestingly, two recent QTL searches in rice for low Cd accumulation in grain from different ecotypes have identified OsHMA3, orthologous to AtHMA3, as responsible for Cd sequestration in the rice roots leading to a decrease Cd content in grain[17,18]. *AtHMA3* was found further as the major locus responsible for the variation in leaf Cd accumulation observed in a diverse population of 349 *A. thaliana* accessions[19].

This corpus of data suggests that genetic engineering of the expression levels of *AtHMA2, AtHMA3* and *AtHMA4* orthologues in agronomic species should have a large potential of applications in biotechnologies such as Zn biofortification and Zn and Cd phytoremediation. However, as underlined by Palmgren and *col*.[6] the involvement of these transporters together in Zn and Cd transport can be problematic in biofortification efforts of food crops in which the accumulation of toxic compounds such as Cd would be detrimental. Recently, different *HMA4* genes have been tested as potential tools for engineering Zn content in various species leading to contrasted results. Ectopic expression of *AtHMA4* in tobacco[20] led to plants more sensitive to Zn toxicity, but less to Cd. Zn accumulation was affected too, but the effects were tightly dependent on the metal concentration in the medium. Expression of *AhHMA4* in tobacco[21] and in tomato plants[22] under the control of its native promoter expected to trigger a high expression level resulted in higher Zn absorption and accumulation in the upper parts. In tobacco this transgene lowered Cd accumulation, while in tomato it triggered a Zn overload of the apoplast and a Fe deficiency response. In a recent study, Mills and *col.*[23] have introduced *HvHMA2* from barley in the *Athma2* A*thma4* mutants *of Arabidopsis. HvHMA2* was able to rescue the stunted phenotype of the *hma2 hma4* double mutants but Zn content increase was limited to 10 to 20% of the Zn content found in wild type plants and seeds. Despite this low level of Zn, *35S::HvHMA2 Athma2 Athma4* triple mutant plants were able to produce viable seeds. Thus, expression of specific *HMA4* in other species generally led to rather unpredictable results and reveals the complexity of the transfer of a heterologous physiological process without interacting with the native system. In conclusion, crop metal biofortification through manipulation of metal transporters still appears as a complex approach and a better understanding of the mechanisms underlying metal transfer processes is still required to develop more predictable strategies.

In the present study we focused on AtHMA2, AtHMA3 and AtHMA4 transporters in *A. thaliana* and on their potential interest in Zn and Cd phytoremediation and Zn biofortification in seeds. Our results confirm the interest of *AtHMA4* as an efficient tool to manipulate Cd and Zn translocation from root to shoot. When plants were grown on a heavy polluted soil containing large amounts of Zn and Cd, overexpression of *AtHMA4* under the *35S::* promoter resulted in a 3-fold increase in the rosette content of both metals. Concerning Zn biofortification, in an unexpected way Zn seed content was decreased in *AtHMA4* overexpressing plants indicating that a greater Zn content in the shoot is not sufficient to promote an increased Zn content in seeds.

Materials and methods

Plant material

T-DNA mutant lines were obtained from the Department of Genetics of the University of Melbourne[10]. *35S::AtHMA3* mutant lines (*35J, 38F, 36A, 27L*) were obtained by agro-transformation in the laboratory as described in Morel *et al.*[16] while *35S::AtHMA4* lines (*5F, 5H, 5J*) were issued from the lineage of the 5B line described in Verret *et al.*[13] (see Tab. S1).

Hydroponic cultures

Arabidopsis plants were grown in a controlled environment (8-h photoperiod at 300 µmol m^{-2} s^{-1}, 21°C, and 70% relative humidity) in a nutrient solution [800 µM $Ca(NO_3)_2$, $4H_2O$; 2 mM KNO_3; 1.1 mM $MgSO_4$, 7 H_2O; 60 µM K_2HPO_4; 700 µM KH_2PO_4; 20 µM $FeSO_4$, $7H_2O$; 20µM Na_2EDTA, $2H_2O$; 75 nM $(NH_4)Mo_7O_{24}$, $4H_2O$; 3.5 µM $MnSO_4$, H_2O; 3 µM $ZnSO_4$, $7H_2O$; 9.25 µM H_3BO_3; 785 nM $CuSO_4$, 5 H_2O; final pH 5.8] with additional 1% (w/v) sucrose and 0.8% (w/v) bacto-agar in the case of the solid medium. The germination of surface-sterilized seeds of wild-type and mutant lines was carried out on solid medium. After 2 weeks, the plantlets were placed on sand, left there for an additional 2-week period, and finally transferred to a home-built hydroponic culture setup. The nutrient solution and the toxic metal were replenished every 2 days; Cd was supplied from a 100 mM $CdCl_2$ stock solution.

Elemental analyses

Hydroponically grown plants were harvested after 11 days of metal treatment. Roots were rinsed with 10 mM EDTA and then with distilled water. Roots and leaves were dried for 48 h at 50°C and mineralized as described in Morel *et al.*[16]. The metal content of these

samples was determined using ICP-AES (Vista MPX; Varian). The contaminated soil was homogenized using a concrete mixer and its metal content characterized by analyzing 8 samples by ICP-AES. Elemental analyses of seeds were performed on seeds from plants cultivated on soil and watered every four days with half-Hoagland nutrient solution with or without 10µM $CdCl_2$.

Radiolabelling experiments

Seeds were germinated on soil and then transferred on sand moistened daily with Coïc-Lesaint nutrient medium. The plants were grown in a controlled environment with the following conditions: 8 h day length, 300 µmol photons m^{-2} s^{-1}, 21°C day / 18°C night and 60 – 70% relative humidity. After 5 to 6 weeks, plants were removed from sand and transferred to hydroponic vessels filled with the nutrient solution for 2 days before labelling.

Time-dependent kinetics of ^{109}Cd and ^{65}Zn transfer to the shoot

Fifty mL FALCON tubes were filled with the nutrient solution (without Zn). For ^{109}Cd labelling, Zn was adjusted to 3 µM and 3 µM $CdCl_2$ were added to the medium and the specific activity of ^{109}Cd ($CdCl_2$, from Amersham) was adjusted to 100 Bq ml^{-1}. One plant per tube was labelled during 1 h. Roots were then rinsed for 5 min in 5 mM $CaCl_2$ and the plants were transferred back to the hydroponic vessel. After different times (3h to 5 days), some plants were harvested. Roots and shoots were separated, dried overnight at 60°C and weighted. The specific activity of the whole roots and the whole shoots were measured using a Wizard 1480 gamma counter (Perkin). The results were expressed as a ratio of shoot activity to the whole plant activity (shoot and roots). For ^{65}Zn labelling, Zn was adjusted to 3 µM ($ZnSO_4$). Specific activity of ^{65}Zn ($ZnCl_2$, from CERCA-LEA, Pierrelatte, France), was also adjusted to 100 Bq ml^{-1}, then the protocol was identical to the one described for ^{109}Cd labelling.

In a specific series of experiments to investigate a competition between Zn and Cd absorption and translocation, plants were fed for 24h with the nutrient containing 3µM of Zn and Cd and 100 Bq ml^{-1} of ^{65}Zn and ^{109}Cd.

^{109}Cd and ^{65}Zn imaging experiments

For radiotracer imaging, the labelling solutions containing 3 µM Zn and eventually 3 µM Cd were adjusted to a 2 10^2 Bq ml^{-1} specific activity, either for ^{109}Cd or ^{65}Zn. Each plant was labelled during 1 h in a 50 mL tube, rinsed and then transferred back to the hydroponic vessel. At 12 h for Cd and 5 d for Zn, the plants were harvested. The roots and the shoots were then separated and placed over a storage phosphor screen (GE Healthcare) and let in a cassette overnight. The screen was then scanned using a STORM 840 apparatus.

Results and Discussion

Zn/Cd HMAs and Zn/Cd phytoremediation

Root to shoot translocation is an important parameter in phytoremediation. First, on a physiological point of view Cd translocation is generally observed in hyperaccumulator species and seems to be associated with Cd tolerance through a detoxification of roots; translocation of Cd to the shoot which has a higher rate of biomass synthesis allows a dilution of the toxic in expanding tissues. Second, on a technical point of view Cd transfer to the leaves facilitates the harvest and recycling of contaminated biomass. AtHMA2 and AtHMA4 have been characterized as the main contributors to Zn translocation from root to shoot since a double mutant; *Athma2 Athma4* exhibited a strong phenotype of Zn deficiency[10]. AtHMA2 and AtHMA4 were further characterized as contributing also to Cd transport towards the shoot[10,11]. These initial studies have been performed using very low Cd concentrations in the nutrient solution (below 0.5 µM) and we first checked whether these transporters are efficient along a larger domain of Cd concentrations. ^{65}Zn and ^{109}Cd pulse experiments and hydroponic cultures studies were used to characterize the respective role of AtHMA2 and AtHMA4 in Zn and Cd transport *in planta*.

Col has a higher rate of ^{65}Zn and ^{109}Cd translocation than Ws

Col, Ws, *Athma2*, *Athma4* and *35S::AtHMA4* plants were fed for 1h with ^{109}Cd or ^{65}Zn radioisotopes at an equimolar ratio of 3 µM in the nutrient solution and distribution of the radioisotopes in the root and shoot tissues were followed during 6 days. Both elements reached a plateau after about 5 to 6 days (Fig. 1) and when experiments were continued for 2 weeks there was no evidence for a shoot to root redistribution of Zn and Cd (data not shown). Concerning the wild type genotypes, Col was more efficient than Ws in ^{65}Zn and ^{109}Cd translocation from root to shoot by 27% and 88%, respectively after three days (Fig.1). This more efficient translocation could be related to the fact that in Col a premature stop codon in the *AtHMA3* gene led to the synthesis of a likely inactive polypeptide invalidating Cd and Zn storage into root vacuoles and thus increasing their translocation to the shoot (Morel e*t al.*, 2009). After five to six days ^{109}Cd or ^{65}Zn reached a plateau phase in the shoot that continued for at least two weeks, suggesting that there was no shoot to root transfer of Zn and Cd during the vegetative phase.

*At*HMA4 *is the main actor in Zn and Cd translocation*

Among the mutants and overexpressing lines, the different genotypes displayed important differences in the partitioning of the radioisotopes (Fig. 1). *AtHMA4* disruption had the highest impact by decreasing ^{109}Cd and ^{65}Zn translocation by at least 50% (Fig. 1A, 1C). In contrast, *AtHMA4* overexpression led to an increased translocation of both radioisotopes (Fig. 1B, 1D) in accordance with our previous work[13]. In contrast, At*HMA2* disruption had no major impact on the translocation of both Zn and Cd (Fig. 1A, 1C). Such impact of *AtHMA4* disruption was also observed when the plants where fed for 24h with 3µM Zn and Cd, leading to a 35% and 65% decrease in Zn and Cd content in the shoot, respectively (data not shown). Autoradiographies of rosette leaves and roots were also performed after feeding the plants for 1h with the radioisotopes and observed 24h or five days later for ^{109}Cd and ^{65}Zn respectively (Fig. 2 A&B). These autoradiographies confirmed the higher rate of translocation of ^{109}Cd in the Col ecotype compared to Ws. While At*HMA2* disruption had no major effect on the translocation of ^{109}Cd to rosette leaves, *AtHMA4* disruption had a visible impact on the

translocation rate (Fig. 2A). As previously observed during pulse experiments, overexpression of *AtHMA4* resulted in an increased translocation of ^{109}Cd (Fig. 2A) and ^{65}Zn (Fig. 2B) to rosette leaves.

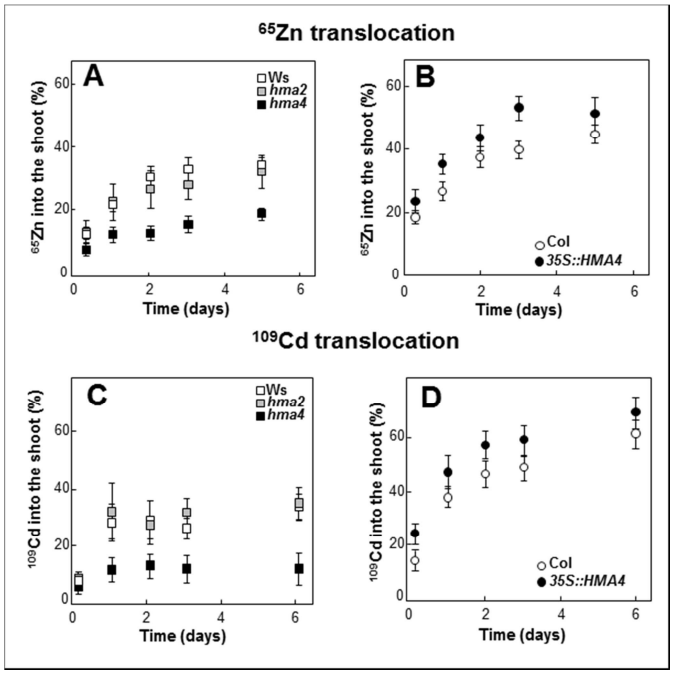

Fig. 1 Percentage of ^{65}Zn and ^{109}Cd into the shoot following a one hour root exposure to the radiotracers **A**) Zn translocation in Ws and *Athma2* and *Athma4* in the same ecotype, Zn in the nutrient solution was 3 µM **B**) Zn translocation in Col and the *35S::AtHMA4-5B* line in the same ecotype, Zn in the nutrient solution was 3 µM **C**) Cd translocation in Ws and the A*thma2* and *Athma4* mutants in the same ecotype, Zn and Cd concentrations in the nutrient solution were 3 µM **D**) Zn translocation in Col and the *35S::AtHMA4-5B* line in the same ecotype, Zn and Cd concentrations in the nutrient solution were 3 µM. Values are the mean of at least 3 experiments; error bars represent standard error to the mean.

^{109}Cd appeared as punctuated at the leaf surface (Fig. 2A), reminding the distribution of trichomes in *A. thaliana* leaves. The Cd content appeared higher in the roots of the Ws genotype compared to the Col one; however HMAs mutations or overexpression did not greatly affect root Cd content (Fig. 2A).

The different genotypes were also grown in hydroponic conditions and after three weeks subjected for eleven days to various Cd concentrations in the medium and Cd content in the rosette leaves was then determined (Fig.3). In the wild type Cd concentration in the rosette was roughly linearly related to the Cd concentration in the medium. Cd was actively transferred to rosette leaves since at equimolar concentrations of Zn and Cd (3 µM), Cd content in the leaves was about twice the Zn content. *AtHMA2* mutation did not impact on Cd content in the rosette while *AtHMA4* mutation had a major effect at low Cd concentrations, *i.e.* a sixty per cent decrease in Cd translocation at 0.5 µM Cd. The effect of the mutation was reduced at higher Cd concentrations, dropping to 20% at 10 µM (Fig. 3A inset). Thus, *AtHMA4* behaves *in planta* as a high-affinity Cd transporter in the micromolar range and when Cd concentration in the medium exceeds 3 µM other transporters seem able to take in charge the translocation of Cd. Additionally, it has been proposed that an apoplastic transport of Cd could take place at these higher concentrations.

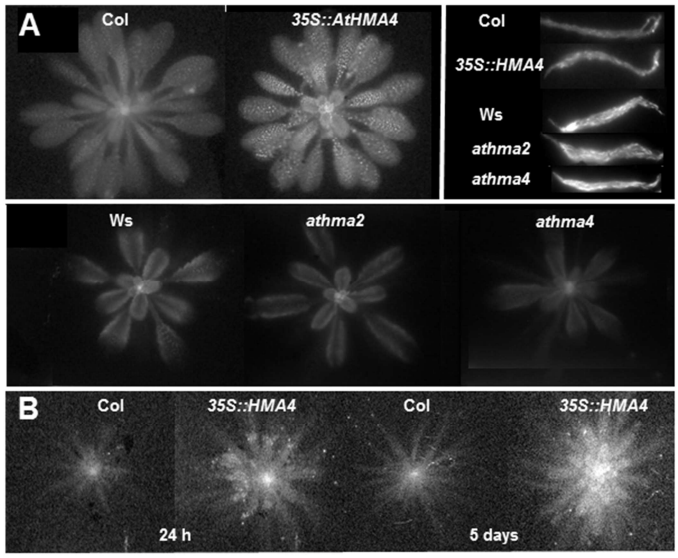

Fig. 2 Autoradiography of representative roots and rosette leaves of wild type plants and mutant or overexpressing lines after labelling with ^{109}Cd or ^{65}Zn. **A**) Autoradiography 24h after 1h of ^{109}Cd labelling of Col and *35S::AtHMA4* line (Col background) and Ws and the *Athma2* and *Athma4* mutants (Ws background) **B**) Autoradiography of ^{65}Zn 24h and 5 days after 1h labelling in the Col and *35S::AtHMA4-5B* line (Col background).

The absence of effect of *AtHMA2* mutation is intriguing since *AtHMA2* and *AtHMA4* display rather similar patterns of expression[10,13] and that a strong phenotype of Zn deficiency was only found in *Athma2 Athma4* double mutants. However these results are in accordance with the previous studies from Hussain and *col.* (2004). In another study *Athma2* mutants were found to have a higher Zn content in the rosette than wild type plants[24]. One explanation would be that *AtHMA4* is overexpressed in *Athma2* mutants but we failed to found such deregulation for *AtHMA4* in *Athma2* mutants using Q-PCR (data not shown). Another explanation could be that the function of *AtHMA2* is to partly contribute to Zn translocation to the shoot and mainly to distribute Zn and by homology Cd in the leaves, allowing their recycling through phloem loading. However, during the pulse experiments ^{109}Cd and ^{65}Zn reached a plateau phase in the shoot after five to six days (Fig. 1) that continued for at least two weeks (data not shown), suggesting that there was no further shoot to root transfer of Zn and Cd.

We further checked whether Cd may impact on Zn translocation through a competitive mechanism. A competition between Zn and Cd translocation was expected since AtHMA4 behave as a Zn/Cd transporter in heterologous expression experiments[25,26]. Such competition was observed on the short term using ^{109}Cd and ^{56}Zn, the uptake of Zn being decreased by about fifty percent in the presence of an equimolar concentration of Cd (Fig. S1). However,

when Cd was applied on a longer period such as eleven days it did not impact on the rosette Zn content of wild type and *Athma2* mutants (Fig. 3B) despite Cd content was higher than Zn content in the rosette when Zn and Cd were given at an equal concentration of 3 µM. These results suggest that Zn content in rosette leaves is under a strong homeostatic process and that the apparent absence of competition in translocation is certainly due to an adaptation of the plant in the presence of Cd. Disruption of *AtHMA4* led to a fifty to thirty percent decrease in Zn content of the rosette in the absence as well as in the presence of Cd (Fig. 3B).

All these data point to AtHMA4 as an interesting tool for phytoremediation since it is the major actor in Cd translocation, its overexpression increases the Cd content in the plant upper parts and the presence of Cd in the upper parts does not greatly impact the Zn content.

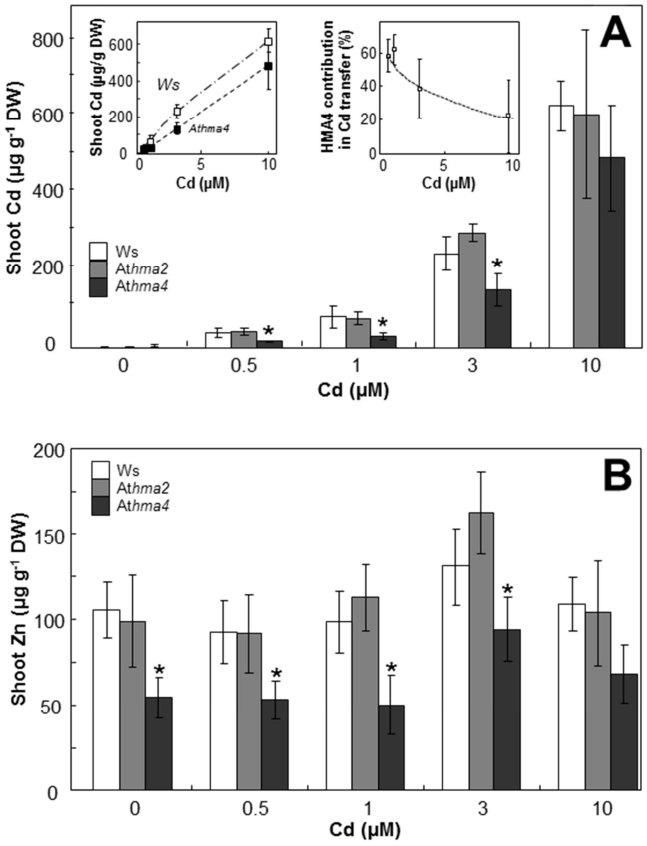

Fig. 3 Shoot Cd and Zn content, after eleven days in hydroponic conditions in presence of various Cd concentrations. **A**) Cd content in rosette leaves of Ws, *Athma2* and *Athma4* mutants. Left inset: shoot Cd content according to Cd concentration in the nutrient solution. Right inset: contribution of *AtHMA4* to Cd translocation according to Cd concentration in the nutrient solution. **B**) Zn content in rosette leaves of Ws, *Athma2* and *Athma4* mutants according to Cd concentration in the nutrient solution. Values are the mean of at least 3 experiments; error bars represent standard error to the mean. Asterisk $p<0.05$.

Overexpression of *AtHMA4* increases the potential of Zn and Cd extraction from a metal polluted site

Wild type plants and two lines overexpressing *AtHMA4* were grown on a polluted soil harvested in Mortagne du Nord where a lead smelter called "Metaleurop Nord" has been the source of a large pollution[27]. Emissions from the industrial smokestacks were estimated to 4 t of Cd and 67 t of Pb per year during the eighties. The industrial sites closed in 2003, leaving a large contaminated area of spoiled agricultural and urban soils. Since then this area is used to experiment different strategies of remediation. Physicochemical properties of the soil used in the present have already been described[28] (soil S2). We observed a high concentration of heavy metal, mainly Zn and Pb in this soil (Fig. 4). *A. thaliana* from the Col ecotype and two lines overexpressing *AtHMA4* under the control of the strong *35S::* promoter called *5B* and *5H* (in the Col background) were grown for 6 weeks on this soil and were just watered with tap water. At the end of the culture the weight of plants were almost identical, the *5B* and *5H* transgenic lines being only about 10% heavier than wild type plants. However the two overexpressing lines were able to accumulate in the rosette leaves more than two fold the Zn content and almost three fold the Cd content found in the wild type (Fig. 4). Such increase in Cd and Zn uptake is interesting since one of the main problems of phytoremediation is generally the time needed to recover a clean soil which is directly related to the biomass production and to the translocation capabilities of the plants.

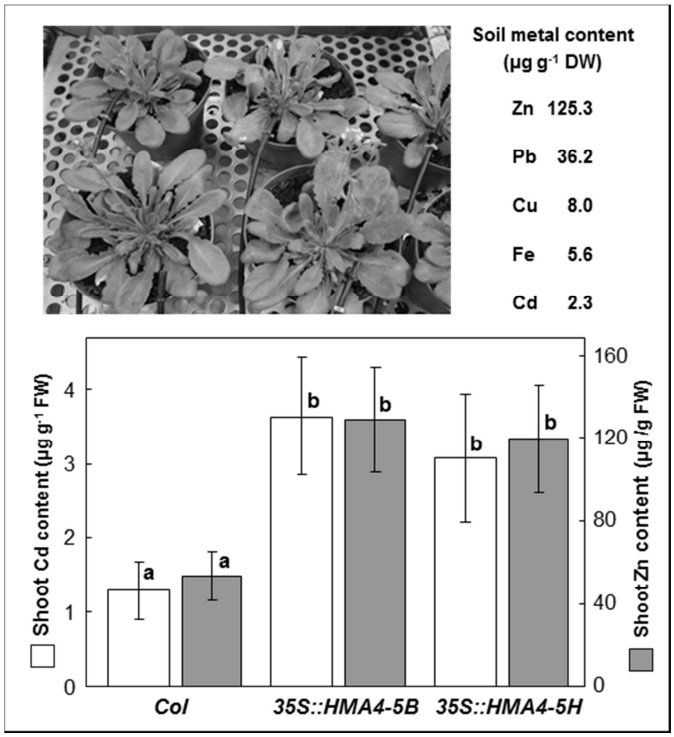

Fig. 4 Shoot Cd and Zn contents of Col, *5B* and *5H*, two lines overexpressing *AtHMA4* under the control of the *35S::* promoter. Plants were grown for four weeks on a Zn and Cd contaminated soil coming from an area polluted by lead and zinc smelters. Values are the mean of at least 3 experiments; error bars represent standard error to the mean; a was significantly different from b, $p<0.05$.

Expression levels of Zn/Cd *HMAs* and Zn content in seeds

As exposed in the introduction, improving crops to raise food Zn

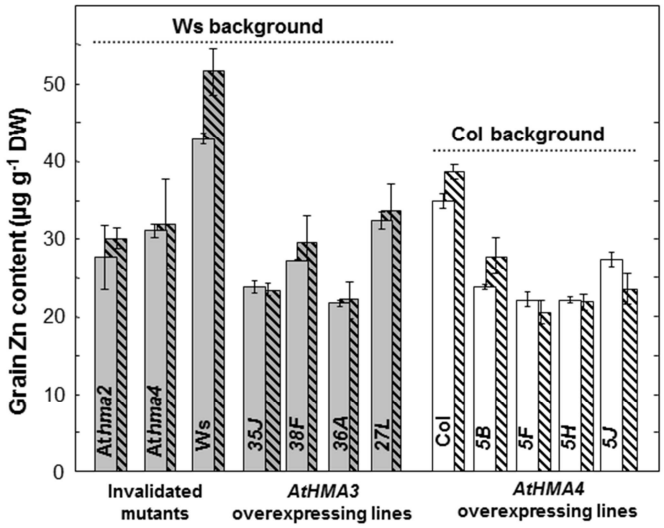

Fig. 5 Zn content in seeds from plants invalidated for *AtHMA2*, *AtHMA3* and *AtHMA4* or overexpressing *AtHMA3* or *AtHMA4*. White columns, Col background, grey columns Ws backgrounds. Dashed bars, identical conditions but 10 µM $CdCl_2$ was added to the nutrient solution. Values are the mean of at least 4 to 7 different plants per genotype. Error bars represent standard error to the mean, $p<0.05$.

content in the edible parts would be an elegant and cost-effective solution to Zn deficiency in the human diet[6,7]. We took advantage of the numerous lines either invalidated or overexpressing *AtHMA3* or *AtHMA4* to test their efficiency in grain Zn biofortification. Seeds were harvested from wild type and mutant plants grown in pots of loam watered every four days by flooding the pots in a half-strength Hoagland's nutrient solution for thirty minutes. In these conditions of high Zn availability none of the plants presented a visible phenotype. Seeds were mineralized and their cation content determined. Zn content was found 28% higher in the Ws accession than in the Col one (Fig. 5). As expected, invalidation of the main actors in Zn xylem loading, *AtHMA2* or *AtHMA4*, resulted in about a thirty percent decrease in Zn content in seeds.

Four lines overexpressing *AtHMA3* in the Ws background displayed a reduced Zn content in seeds by about forty percent compared to the wild type accession. This can be explained by the fact that *AtHMA3* is in Arabidopsis and rice a vacuolar pump that allows the uptake of Zn and Cd from the cytoplasm into the vacuole[16, 17,18], thus overexpression of such transporter is expected to decrease the seeds Zn content. More surprising was that the four lines overexpressing *AtHMA4* also displayed a decreased Zn content in seeds. Despite these lines were found to have a higher potential of Zn translocation from the roots to the shoot[13]. Overexpression of *AtHMA4* resulted in mean to a thirty percent decrease in seeds Zn content. This observation suggests that Zn content in the rosette is not the major determinant of Zn filling in seeds. Ectopic expression of *AtHMA4* could result in a widespread distribution of Zn in leave tissues which has an adverse effect on grain filling. Tauris and *col.*[29] have shown using laser capture microdissection that in barley *HvHMA2*, homologous to *AtHMA2* and *AtHMA4*, is the only Zn/Cd *HMA* member expressed in seeds and solely at the level of transfer cells. Thus, it is likely that ectopic expression of *AtHMA4* results in a competitive transfer that diminishes the targeting of Zn to seeds. While *AtHMA4* certainly stays an interesting candidate for Zn biofortification strategies, a considerable work stays to be done around the promoters which should be used to optimize the targeting of Zn to the seeds.

As underlined in previous reviews on Zn biofortification[6], a potential risk of the use of Zn/Cd P_{1B}-ATPases is that an increase in Zn content in the grain could be accompanied by a concomitant increase in the highly toxic heavy metal Cd. This hypothesis was tested using the lines previously studied and using a similar protocol with the difference that the nutrient solution used to flood the pots was containing 10 µM $CdCl_2$ still in the presence of 3 µM Zn. A toxic effect of Cd treatment was clearly observed since it resulted in a mean 28% decrease in seeds production for the twelve lines studied. However this treatment did not impact greatly the Zn content in seeds in all genotypes tested (Fig. 5). In these conditions Cd was undetectable in seeds, below the detection limit of ICP-AES for this element meaning that Cd content was lower than 0.5 µg g^{-1} DW in *A. thaliana* seeds. This observation questions the use of Arabidopsis as a model plant to work on Cd translocation to the grain. As a comparison, Cd content in brown rice was in the range of 6 µg g^{-1} DW when the plants were challenged with 20 µM Cd[17]. Moreover, it has been recently published that OsHMA2, orthologous in rice to AtHMA4 in Arabisopsis, is involved in Zn and Cd upward translocation and in grain Cd filling while Zn filling appears to be driven by another transporter[30]. Transporters from the Nramp family could be good candidates[31,32] and OsNRamp5 has already been clearly identified as a major contributor to Cd accumulation in rice grain[33].

Conclusions

Phytoremediation and biofortication are emerging green technologies that are still in their early developmental stages. Full scale applications using phytoremediation are limited at the exception of phytostabilization that consists in the establishment of a vegetal cover to structure the soil and diminish erosion by wind and water in order to limit the spreading of toxics. A more curative technique for heavy metal polluted soil is phytoextraction which uses the absorption properties of plant roots to extract the metal from the soil[34,35]. Efficiency of this technique is tightly linked to numerous parameters such as biomass production and culture rotation, rate of root uptake which depends on metal bioavailability and root to shoot translocation to harvest the contamination stored in the shoot tissues. This last step is an important limiting factor as in most plants the toxic metal content is higher in root than in shoot at the notable exception of hyperaccumulator species[34,35]. These last years, molecular genetic studies have shown that *AtHMA4* orthologues in hyperaccumulators have been submitted to duplications and driven by strong promoters and that they play a crucial role in loading Zn and Cd into the xylem[36,15]. In the present study we observed that ectopic expression of *AtHMA4* in Arabidopsis conducted to a 3-fold increase in the extraction of Zn

and Cd from a soil contaminated by metallurgic activity. This is an important result since a higher rate of translocation means a reduction in the time needed for soil remediation which is the main drawback of phytoremediation which can last for decades[34,35]. This study points also that expression of homologous HMAs may be crucial to get an enhanced activity. We propose the hypothesis that HMAs function as multimers and that expression of a heterologous gene could result in non-functional multimeric transporters through the formation of dominant negative complexes. Such hypothesis of positive or negative interactions between proteins with close structures could explain the large diversity of responses when heterologous *HMAs* were expressed in various species[21,22,20,25,37]. This should be taken into account by either overexpressing endogenous *HMAs* or by evaluating the compatibility of isoforms from different species through co-expression in yeast.

Many efforts have been made to understand the molecular basis of the selectivity and velocity of HMAs. These studies have frequently focused on the characteristic peptide extensions exhibiting heavy metal chelation properties. Manipulation of these extensions can result in a higher velocity of the transporter as well as a higher metal specificity[38,39]. However, a second point raised by this study is that an important work has to be done concerning the selection of promoters according to the objectives. While ectopic expression of *AtHMA4* using the strong constitutive *CAMV* promoter seems interesting in the frame of phytoremediation this study shows that it has an adverse effect on seeds Zn content. It is somehow logical that the large spreading of Zn in the rosette leaves induced by overexpression of *AtHMA4* is detrimental to a correct targeting of Zn towards the seeds through a competitive mechanism. A possibility would be to use promoters driving the expression of macro-element transporters that share the expression domain of the *AtHMA4* gene in the stele such as the *SKOR* promoter[40] driving the expression of an outward potassium channel feeding K^+ into the xylem or *PHO1* promoter[41], which deserves the same function for PO_4^-. This may result in a higher rate of transport while keeping the same pathway of transport to the seeds. In conclusion *AtHMA4* is certainly an interesting biotechnological tool for phytoremediation but concerning biofortification its implementation will need further progresses.

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Notes

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Bibliographic References

1 J. M. Berg, Y. Shi, *Science*, 1996, **271**:1081-5.
2 J.E Coleman, *Curr. Opin. Chem. Biol.* 1998, **2**:222–234.
3 W. Maret, H. H. Sandstead, *J. Trace Elem. Med. Biol.*, 2006, **20**:3-18.
4 P. J. White, M. R. Broadley, *New Phytol.*, 2009, **182**:49-54.
5 D. Cohen, *New Scientist*, **2007, 2605**: 34–41.
6 M. G. Palmgren, S. Clemens, L. E. Williams, U. Krämer, S. Borg, J. K. Schjørring, D. Sanders, *Trends Plant Sci.*, 2008, **13**:464-473.
7 P. J. White, M. R. Broadley, *New Phytol.*, 2009, 182:49-54.
8 J. L. Hall, L. E. Williams, *J. Exp. Bot.*, 2003, **54**: 2601-2613.
9 N. Grotz, M. L. Guerinot, *Biochim. Biophys. Acta*, 2006, **1763**:595-608.
10 D. Hussain, M. J. Haydon, Y. Wang, E. Wong, S. M. Sherson, J. Young, J. Camakaris, J. F. Harper, S. Cobbett, *Plant Cell* 2004, **16**: 1327–1339.
11 C. K. E. Wong, C. S. Cobbett**,** *New Phytologist*, 2009, **181**: 71-78.
12 C. K. E. Wong, R. S. Jarvis, S. M. Sherson, C. S. Cobbett, *New Phytologist*, 2009, **181**: 79-88.
13 F. Verret, A. Gravot, P. Auroy, N. Leonhardt, P. David, L. Nussaume, A. Vavasseur, P. Richaud, *FEBS Letters*, 2004, **576**: 306-312.
14 I. N. Talke, M. Hanikenne, U. Krämer, *Plant Physiol.*, 2006, **142**:148-167.
15 A. R. Craciun, C. L. Meyer, J. Chen, N. Roosens, R. De Groodt, P. Hilson, N. Verbruggen, *J. Exp. Bot.* 2012, **63**:4179-89.
16 M. Morel, J. Crouzet, A. Gravot, P. Auroy, N. Leonhardt , A. Vavasseur, P. Richaud, *Plant Physiol.*, 2009, **149:** 894-904.
17 D. Ueno, N. Yamaji, I. Kono, C. F. Huang, T. Ando, M. Yano, J. F. Ma, *Proc. Natl. Acad. Sci. U S A*, 2010, **107**:16500-160505.
18 H. Miyadate, S. Adachi, A. Hiraizumi, K. Tezuka, N. Nakazawa, T. Kawamoto, K. Katou, I. Kodama, K. Sakurai, H. Takahashi, *New Phytologist.* 2011, **89**:190-9.
19 D. Y. Chao , A. Silva , I. Baxter , Y. S. Huang , M. Nordborg , J. Danku , B. Lahner , E. Yakubova , D. E. Salt, *PLoS Genet.*, 2012, e1002923. doi: 10.1371.

20 O. Siemianowski, R. F. Mills, L. E. Williams, D. M. Antosiewicz, *Plant Biotechnol. J.*, 2011, **9**:64-74.

21 A. Barabasz, U. Kramer, M. Hanikenne, J. Rudzka, D. M. Antosiewicz, *J. Exp. Bot*, 2010, **61**: 3057-67.

22 A. Barabasz, A. Wilkowska, A. Ruszczyńska, E. Bulska, M. Hanikenne, M. Czarny, U. Krämer, D. M. Antosiewicz, *Physiol. Plant.*, 2012, **145**:315-31.

23 R. F. Mills, K. A. Peaston, J. Runions ,L. E. Williams, *PLoS One*, 2012, **7**: e42640.

24 E. Eren, J. M. Argüello, *Plant Physiol*., **2004, 136**:3712-3723.

25 R. F. Mills, G. C. Krijger, P. J. Baccarini, J. L. Hall, L. E. Williams, *Plant J.*, 2003, **35**:164-76.

26 F. Verret, A. Gravot, P. Auroy, S. Preveral, C. Forestier, A. Vavasseur, P. Richaud, *FEBS Letters*, 2005, **579**:1515-1522.

27 F. Douay, H. Roussel, C. Pruvot, A. Loriette, H. Fourrier, *Science of The Total Environment*, 2008, **401**: 29-38.

28 A. Schneider, *Environ. Sci. Technol*. 2008, **42**:4076–4082

29 B Tauris, S Borg, P. L. Gregersen, P. B. Holm, *J. Exp. Bot.*, 2009, **60**:1333-47.

30 R. Takahashi, Y. Ishimaru, H. Shimo, Y. Ogo, T. Senoura, N. K. Nishizawa, H. Nakanishi, *Plant Cell Environ.*, 2012, **35**:1948-57.

31 S. Thomine, R. Wang, J. M. Ward, N. M. Crawford , J. I. Schroeder JI., *Proc Natl Acad Sci U S A.*, 2000, **97**:4991-6.

32 M.J. Milner, N. Mitani-Ueno, N. Yamaji, K. Yokosho, E. Craft, Z. Fei, S. Ebbs, M. Clemencia Zambrano, J. F. Ma, L.V. , *Plant J.*, 2014, **78**:398-410.

33 Sasaki, N. Yamaji, K. Yokosho, J. F. Ma, *Plant Cell*, 2012, **24**:2155-67.

34 D. E. Salt , R. D. Smith , I. Raskin , *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 1998, **49**:643-668.

35 E. Pilon-Smits, *Annual Review of Plant Biology*, 2005, **56**: 15-39.

36 M. Hanikenne, I. N. Talke, M. J. Haydon, C. Lanz, A. Nolte, P. Motte, J. Kroymann, D. Weigel, U. Krämer, *Nature*, 2008, **453**:391-5.

37 M. Iqbal, I. Nawaz, Z. Hassan, H. W. Hakvoort, M. Bliek, M. G. Aarts ,H. Schat, *Front Plant Sci.*,2013, doi: 10.3389/fpls.2013.00404.

38 L. Baekgaard, M.D. Mikkelsen, D.M. Sørensen, J.N. Hegelund, D.P. Persson, R.F. Mills, Z. Yang, S. Husted, J.P. Andersen, M.J. Buch-Pedersen, J.K. Schjoerring, L.E. Williams, M.G. Palmgren, *JBC*, 2010, **41**:31243-52

39 R.F. Mills, B. Valdes , M. Duke , K.A. Peaston , B. Lahner , D.E. Salt , L.E. Williams, 2010, *Plos One*, **5**(10): e13388.

40 F. Gaymard, G. Pilot, B. Lacombe, D. Bouchez, D. Bruneau, J. Boucherez, N. Michaux-Ferrière, J. B. Thibaud, H. Sentenac, *Cell*, 1998, **94**:647-55.

41 D. Hamburger, E. Rezzonico, J. MacDonald-Comber Petétot, C. Somerville, Y. Poirier, *Plant Cell*, 2002, **1**:889-902.

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Supplementary Table 1

Lines	Gene number	Ecotype	Stock Ref.	Described in	Expr. level *vs* wild-type *
hma2-4	At4g30110	Ws	SALK-050924	Hussain *et al.* 2004	nil
hma4-2	At2g10110	Ws	SALK-034393	Hussain *et al.* 2004	nil
35S::HMA4	At2g10110	Col	NASC N9416-17	Verret *et al.* 2004	Fig 44 F. Verret's PhD thesis 2004
35S::HMA3	At4g30120	Ws	request to authors	Morel *et al.* 2009	Tab S2 in Morel et al. 2009

Table S1 Main characteristics of the different lines used in this study. ***** Quantification using ImageJ software estimated an increase in the expression levels by 5.8 – 6.8 fold for the *35S::HMA3* lines and around 6 fold for the *35S::HMA4* lines.

Supplementary Figure 1

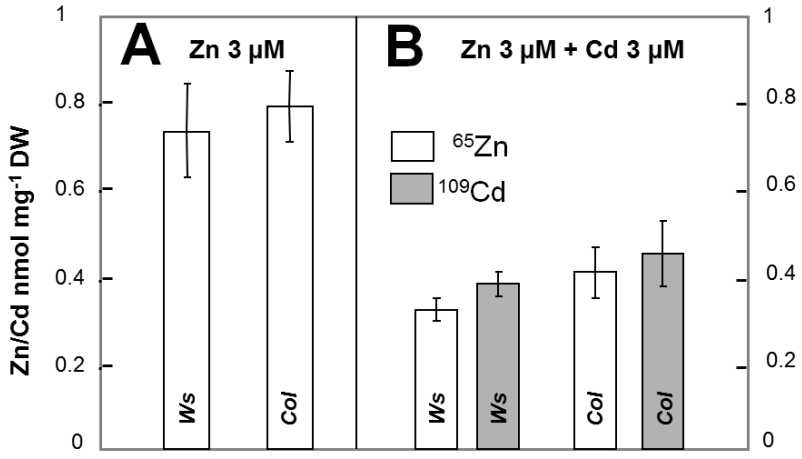


Fig. S1 Daily uptake of Zn (white bars) and Cd (grey bars) when plants were placed in 3 µM Zn (A) or 3 µM Zn plus 3 µM Cd (B) as followed through ^{65}Zn and ^{109}Cd absorption. Values are the mean of at least 3 experiments; error bars represent standard error to the mean.

Graphical Abstract

Changes in the expression levels of P_{1B2}-ATPases in *Arabidopsis* impacts Zn and Cd contents in shoot and seeds

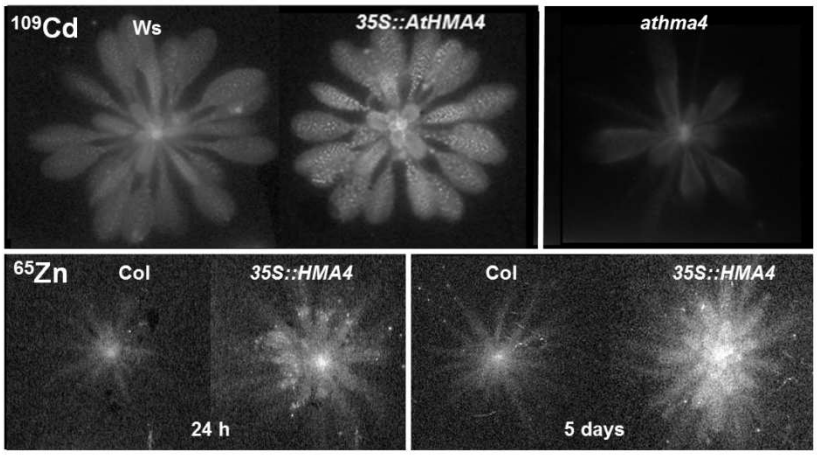
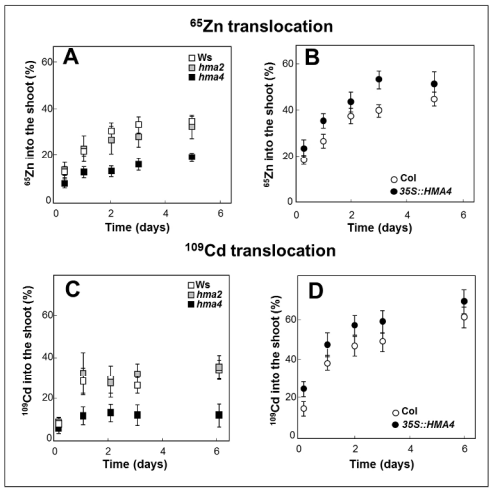


Figure 1



190x254mm (300 x 300 DPI)

Figure 2

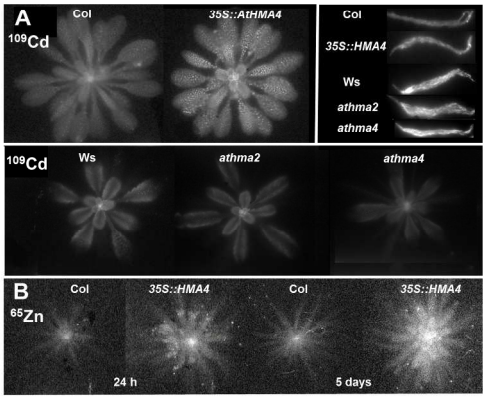


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Figure 3

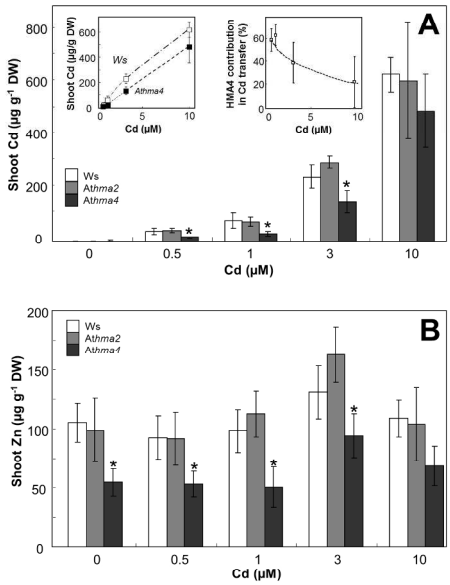

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Figure 4

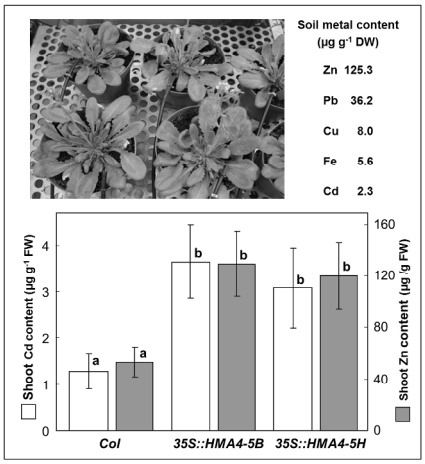

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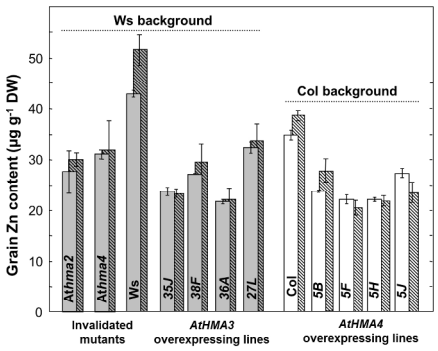
Figure 5



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