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Long-distance transport, vacuolar sequestration and transcriptional responses induced by cadmium and arsenic

David G. Mendoza-Cózatl, Timothy O. Jobe, Felix Hauser, and Julian I. Schroeder*

Division of Biological Sciences, Cell and Developmental Biology Section, University of California, San Diego, La Jolla, California 92093-0116, USA

Summary

Iron, zinc, copper and manganese are *essential* metals for cellular enzyme functions while cadmium, mercury and the metalloid arsenic lack any biological function. Both, *essential* and *non-essential* metals and metalloids are extremely reactive and toxic. Therefore, plants have acquired specialized mechanisms to sense, transport and maintain *essential* metals within physiological concentrations and to detoxify *non-essential* metals and metalloids. This review focuses on the recent identification of transporters that sequester cadmium and arsenic in vacuoles and the mechanisms mediating the partitioning of these metal(loid)s between roots and shoots. We further discuss recent models of phloem-mediated long-distance transport, seed accumulation of Cd and As and recent data demonstrating that plants possess a defined transcriptional response that allow plants to preserve metal homeostasis. This research is instrumental for future engineering of reduced toxic metal(loid) accumulation in edible crop tissues as well as for improved phytoremediation technologies.

Keywords

ABC transporters; Heavy Metal ATPase; transcription factors; phytochelatins; phloem transport; hyperaccumulator plants; phytoremediation

Introduction

The trace metals iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) are *essential* to all organisms functioning as co-factors in a variety of enzymes and proteins [1–3]. Metals are intrinsically very reactive and, therefore, their intracellular concentrations must be tightly regulated. Other metals, such as cadmium (Cd), lead (Pb), chromium (Cr), mercury (Hg) and the metalloid arsenic (As) are toxic and biologically *non-essential*, but can enter plants using the same transporters used for *essential* nutrient uptake [3–6]. Once inside the cell, these toxic *non-essential* metals can displace and interfere with the function of *essential* metals. Therefore, organisms have acquired genetic and biochemical mechanisms to sense, transport and maintain *essential* metals within a non-toxic physiological range (i.e. metal homeostasis), while detoxifying *non-essential* metals (Fig. 1) [1–5,7].

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*Corresponding author, Julian I. Schroeder, Ph D, University of California, San Diego, Division of Biological Sciences, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA., +1 858 534-7759 (phone), +1 858 534-7108 (fax), julian@biomail.ucsd.edu.

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Here we review recent advances in the identification of key genes mediating accumulation and tolerance to cadmium and arsenic, including the identification of the vacuolar phytochelatin transporters that remained elusive for more than 15 years [8••,9••], transporters contributing to metal partitioning between roots and shoots [10••, 11•] and the characterization of vacuolar metal transporters from hyperaccumulator species [12•,13••]. We will discuss recent data demonstrating that the transcriptional changes induced by exposure to *non-essential* toxic metals are part of a transcriptional response to preserve metal homeostasis [14].

Identification of the long-sought vacuolar phytochelatin transporters

Phytochelatin synthesis is perhaps one of the most well studied mechanisms mediating detoxification of Cd, As, Zn, Hg and Cu in plants and some yeast, including *Schizosaccharomyces pombe* [3,5,15]. Phytochelatins (PCs) are glutathione-derived peptides synthesized in the cytosol where they form PC-metal(loid) complexes that are transported into vacuoles, thus removing these toxic elements from the cytosol [3,5,15–16]. More than 15 years ago, research suggested that vacuolar uptake of PC-metal(loid) complexes was mediated by ATP-binding cassette transporters (ABC transporters) [17–19]. In 1995, Hmt1 (Heavy metal tolerance 1), a half-size ABC transporter was identified as a vacuolar PC transporter required for Cd tolerance in the yeast *S. pombe* [17–19]. However, attempts to identify Hmt1-like transporters in plants were unsuccessful. In fact, the availability of diverse plant genome sequences revealed that in several cases the plant genes with the highest similarity to *S. pombe* Hmt1 were the half-size ABC transporters of the mitochondria (ATMs) [16] (Supp. Fig. 1 and Supp. Table 1).

Notably, the precise mechanism by which Hmt1 confers tolerance to Cd was far from understood. Hmt1 confers tolerance to Cd, but not arsenite [As(III)] or arsenate [As(V)] [20–21]. Furthermore, heterologous expression of Hmt1 enhances Cd tolerance in *Escherichia coli* and *Saccharomyces cerevisiae*, organisms devoid of phytochelatins (PCs), suggesting that Hmt1 also mediates tolerance in a PC-independent manner [8••, 20–21]. Subsequent analyses in *S. pombe* demonstrated that vacuoles from the *hmt1* mutant still contained a significant amount of PCs, suggesting the existence of additional vacuolar PC transporters [8••,20]. Following a systematic deletion of vacuolar ABC transporters in *S. pombe* and *Arabidopsis*, two groups have recently, and independently, identified full-length ABC transporters mediating vacuolar PC uptake in *S. pombe* (Abc2) and *Arabidopsis* (MRP1/ABCC1 and MRP2/ABCC2, Fig. 1) [8••,9••]. *S. pombe* Abc2 is able to rescue the cadmium sensitivity and the capacity to accumulate PCs in vacuoles of a mutant devoid of five vacuolar ABC transporters (*hmt1 abc1 abc2 abc3 abc4*) [8••]. On the other hand, the *Arabidopsis* double mutant *abcc1 abcc2* is arsenic hypersensitive, and vacuoles isolated from this mutant show a dramatic reduction in vacuolar PC-As uptake compared to vacuoles isolated from wildtype plants [9••]. Interestingly, ABCC1 and ABCC2 were previously described ABC transporters mediating the sequestration of glutathione-conjugates complexes into plant vacuoles [16,22].

In contrast to Hmt1, a search for related genes in other plant species readily identified ABCC2/Abc2-like proteins throughout the plant kingdom including both terrestrial and aquatic plants (Fig. 2, Table 1). It should be noted, however, that *S. pombe* Abc2 and *Arabidopsis* ABCC2 share $\approx 37\%$ identity at the amino acid level, yet they perform similar functions. Functional analysis together with sequence-similarity searches should be used to positively identify vacuolar PC transporters in other organisms. The overlapping function of ABCC1 and ABCC2 in *Arabidopsis* also explains why forward genetic screens did not identify these transporters. The identification of ABCC1/2 and Abc2 transporters, which

belong to a subfamily of ABC transporters different from Hmt1, marks the end of the long-standing search for the genetic identity of vacuolar PC-metal(loid) transporters.

The mechanism by which Hmt1 confers Cd tolerance is not fully understood. Hmt1 function is GSH-dependent, but at this point it is unclear whether Cd-GS₂ is one of Hmt1 substrates or whether GSH is required to synthesize a more chemically complex substrate [8•,21]. Hmt1 is structurally related to Atm1, which is also a GSH-dependent half-size ABC transporter that exports FeS clusters from the mitochondrial matrix to the cytosol [23–24]. A recent characterization of the cadmium tolerance mediated by Hmt1 in *S. cerevisiae* and *S. pombe* suggested that Hmt1 may transport CdS clusters from the cytosol into vacuoles [8•] and that either GSH or PCs help to stabilize these clusters in a similar way that GSH stabilize FeS clusters in plants and bacteria [25–26]. Detoxification of GSH-coated CdS clusters would explain why Hmt1 can confer Cd tolerance in organisms devoid of PCs [8•].

Long-distance transport of cadmium and arsenic

Distribution of cadmium and arsenic between roots and shoots is a dynamic process driven by root plasma membrane transporters, xylem-loading/unloading and phloem-loading/unloading processes [27–32]. Uptake of metals and arsenic into roots and the xylem have been extensively studied (reviewed by [4–5]). Briefly, Zn and Cd are taken up by ZIP transporters and are loaded into the xylem by the Heavy Metal ATPases HMA2 and HMA4 (Fig. 1). Arsenate [As(V)] is taken up and loaded into the xylem by phosphate transporters [33] while arsenite [As(III)] uptake and translocation is mediated by members of the NIP subfamily of aquaporins [4–5].

Phloem transport, on the other hand, has been less studied, perhaps due to the technical challenges associated with phloem sampling [34–35]. However, transport through the phloem plays a key role in delivering nutrients, including metals, to developing seeds where xylem-mediated transport plays a minor role due to the limited transpiration rate within reproductive tissues [36]. Therefore, understanding phloem transport mechanisms is important to restrict the transport of toxic *non-essential* metals into (edible) seeds while ensuring the accumulation of *essential* nutrient metals. Analyses from various plant species have shown that the main metal-ligand molecules found in phloem sap are nicotianamine, GSH and PCs [27,30•,37–38]. Nicotianamine has been shown to form complexes with Fe, Cu, Zn and Mn [38, 39–40], while GSH and PCs have orders of magnitude higher affinities for the metal(loid)s Cd, Zn, Hg and As(III) [27,41]. Furthermore, extended X-ray absorption fine structure (EXAFS) analysis of seeds in the Cd hyperaccumulator *Thlaspi praecox* shows that 60% of Cd is coordinated with thiol-containing ligands (Fig. 1) [42]. The finding of PCs in phloem sap [27] was unexpected since PCs have long been considered molecules that mediate the transport of metals from the cytosol into vacuoles. However, research revealed long-distance PC transport [43] and liquid chromatography-mass spectrometry analyses revealed high levels of PCs in the phloem sap of *Brassica napus* [27]. Furthermore, energy-dispersive X-ray microanalysis (EDXMA) in *A. thaliana* found significant levels of Cd and sulfur-Cd complexes in the cytoplasm of companion cells (phloem-loading cells) [37], suggesting that thiols mediate long-distance transport of metal(loid)s through the phloem. The plasma membrane transporters that load PC-metal(loid) complexes into the phloem remain unknown (Fig. 1), but cell-specific transcriptome analyses in *A. thaliana* show that phytochelatin synthase (*PCS1*, At5g44070) is most highly expressed in companion cells (phloem-loading cells) ([44•], Supp. Fig. 2A). Companion cells and sieve elements (phloem) are connected through highly permeable plasmodesmata [35]. Therefore, compounds synthesized in companion cells, or transported into companion cells such as GSH or PCs, are likely to enter the phloem for further transport into sink tissues (e.g. seeds and roots, Fig. 1) [35,45–47]. We have analyzed low molecular weight thiols in *Arabidopsis*

seeds and found significant levels of GSH but not PCs (Mendoza-Coatzl & Schroeder, unpublished). This suggests that thiol-Cd detection in seeds [42] may result from glutathione-Cd conjugates and that PC-Cd complexes loaded into the phloem are more likely to be sequestered in root (sink) vacuoles by the phytochelatin transporters ABCC1 and ABCC2. Notably, ABCC1 and ABCC2 transcript levels are expressed on average 3-fold higher in roots compared to shoots ([44••], Supp. Fig. 2B).

But why are phytochelatins transported through the phloem? The current model suggests that PCs may contribute to the movement of toxic metals out of the shoots where they could impair photosynthesis [27,37]. The movement of Na⁺ and K⁺ from shoots to roots has also been observed [48]. Such re-circulating mechanisms would limit the accumulation of metals in shoots. In rice, it has been suggested that Cd could be transferred directly from the xylem into the phloem at the nodes without being unloaded into leaf blades [31••]. An important consequence of this phloem-mediated transport of toxic elements is that they will be available for accumulation in seeds during the seed-filling stage. Understanding the mechanisms of metal(loid) loading into companion cells, phloem unloading and seed filling and whether these mechanisms are differentially regulated during the vegetative to flowering transition could help to ensure a high nutritional value of fruits, seeds and grains.

Mechanisms contributing to hyperaccumulation of cadmium and arsenic

Plants considered cadmium or arsenic hyperaccumulators have the capacity to tolerate high concentrations of cadmium or arsenic while accumulating a significant fraction of these toxic elements in shoots. Phytochelatins mediate the detoxification of Cd, As, Hg and plants deficient in PC synthesis are highly sensitive to these metal(oids). Hyperaccumulators seem to combine PC-dependent mechanisms with additional mechanisms to enhance their tolerance and accumulation capacity [4,49]. Metal hyperaccumulation has been associated with the following four key processes: 1) enhanced metal(loid) uptake in roots, 2) reduced sequestration of metals in root vacuoles, 3) enhanced root-to-shoot translocation and 4) enhanced sequestration of metals in shoot vacuoles (Fig. 1) [4–5,12•,13••]. The physiology of metal hyperaccumulation has been previously reviewed [4] and several publications have described transcriptional differences between hyperaccumulator and non-hyperaccumulator species [4,49–52]. Of particular interest for this review is the recent identification of ACR3 as a vacuolar As(III) transporter in fern [13••] and the characterization of HMA3 in the Cd hyperaccumulator *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) [12•]. The fern *Pteris vittata* has demonstrated the ability to tolerate and hyper-accumulate arsenic in fronds (reviewed by [53]). The isolation of ACR3, a vacuolar transporter that enhances As(III) tolerance and accumulation in fern was recently reported [13••]. RNAi studies showed that fern with reduced expression of ACR3 were As(III) hypersensitive. On the other hand, HMA3 is a vacuolar Heavy Metal ATPase that was identified as highly expressed in the Cd/Zn hyperaccumulator *Arabidopsis halleri* compared to its close relative and non-hyperaccumulator *A. thaliana* [51]. Interestingly, HMA3 in *A. thaliana* (ecotype Col) is a pseudogene, containing a premature stop codon [54–55]. However, HMA3 is functional in the WS ecotype and its deletion promotes Cd sensitivity [54]. Furthermore, HMA3 over expression enhances tolerance and accumulation of Zn and Cd in the WS background [54]. HMA3 was also recently found to be responsible for differences in Cd accumulation in grains of two varieties of rice [10••]. Loss of HMA3 in roots facilitates root-to-shoot translocation of Cd resulting in an enhanced accumulation of Cd in rice grains. HMA3 was also recently found to be a determinant for Cd hyperaccumulation in *Noccaea caerulescens*. The ecotype Ganges shows enhanced tolerance and accumulation of Cd compared to the Prayon ecotype and a major difference between these two ecotypes is a sevenfold increase in the expression of HMA3 throughout the plant [12•]. Expression of TcHMA3 in *Arabidopsis* significantly enhances the accumulation of Cd and to a lesser extent Zn [12•]. Identification

of HMA3 as a novel contributor to the hyperaccumulator phenotype offers a new strategy to increase cadmium tolerance and accumulation capacity of non-hyperaccumulating plants to improve current phytoremediation strategies. The engineering of plants with higher content of *essential* metals to enhance their nutritional value or higher accumulation of toxic metals for phytoremediation purposes will also require a detailed flux analysis of the metabolic pathways mediating tolerance (i.e. thiol biosynthesis), together with the simultaneous expression of thiol-metal and metal transporters to manipulate tolerance, accumulation and allocation of metals throughout the plant [56–57].

Transcriptional regulation mediated by cadmium and arsenic

Plants exposed to toxic metals or to elevated concentrations of *essential* metals display significant changes in gene expression that allow them to survive sub-optimal growth conditions. The regulatory mechanisms, transcription factors (TFs), and networks mediating these transcriptional responses remain largely unknown. The only transcription factor that has been shown to play a direct role in a Cd-induced transcriptional response in plants is the wheat gene *TaHsfA4a* [58•]. This heat-shock transcription factor was shown to up-regulate metallothioneins (MT's) following Cd exposure in both yeast and rice. Specific residues in the DNA binding motif of *TaHsfA4a* were shown to be critical for Cd-dependent regulation of MT's and are highly conserved in monocot species but are not present in *Arabidopsis* and other dicot species [58•]. Thus, the transcriptional regulators important for metal(loid)-induced transcriptional responses in dicots, including *Arabidopsis*, and metal(loid) hyperaccumulating species remain elusive.

A limited number of transcriptional profiling studies in *Arabidopsis* have shed light on the transcriptional networks that are affected by Cd and As exposure. Time course experiments using low (5 μ M) and high (50 μ M) Cd treatments revealed that rapid Cd-induced gene expression was not correlated with Cd accumulation in the tissues analyzed [14]. These studies also show that Cd-induced gene regulation affects a broad functional range of genes. Within hours of Cd exposure many genes involved in photosynthesis and glucosinolate biosynthesis are downregulated, while genes involved in sulfur metabolism, cell wall metabolism, and phenylpropanoid metabolism are rapidly induced [14,52]. Transcriptional regulators controlling the sulfur-limitation response were not identified in these studies despite a partial overlap between Cd and sulfur deficiency signaling [14,59–61].

As discussed above, Heavy Metal ATPases (HMA) have been identified as key transporters mediating translocation and storage of Cd and Zn (Fig. 1) [54,62–63]. However, it remains unknown how these transporters are regulated at the transcriptional level. Compared to *A. thaliana*, *A. halleri* contains two additional copies of the HMA4 gene as well as numerous alterations in the promoter element leading to higher expression compared to *A. thaliana* [62,64]. In contrast to AtHMA4, AtHMA3 has a very low transcript level and is only slightly up-regulated by Cd exposure in *A. thaliana* [50–51,65]. However, the *A. halleri* ortholog AhHMA3 shows higher expression and is up-regulated by Cd exposure [50–51,65]. These differences suggest that HMA genes are regulated at the transcriptional level but the Cd-induced response pathway and the transcription factors mediating this Cd-induced expression have yet to be identified.

Another transporter that is highly regulated under Cd stress is the low-affinity nitrate transporter NRT1.8 (At4g21680). NRT1.8 was identified as a nitrate transporter that mediates nitrate removal from xylem sap [11•]. Loss of NRT1.8 function mutants are Cd sensitive and accumulate nitrate in the xylem sap. Furthermore, the *nrt1.8-1* mutant accumulates nitrate in roots during Cd exposure compared to the wild-type control [11•]. Microarray experiments identified NRT1.8 as being one of the most highly up-regulated

transporter genes in roots under Cd stress. The transcript level of this transporter was shown to increase in a concentration-dependent and time-dependent manner after cadmium exposure [11•]. The NRT1.8 transporter is the first gene directly linking nitrogen metabolism to Cd-stress response and supports previous models where nitrogen-containing compounds are partially responsible for the translocation of Cd from roots to shoots through the xylem.

It is also important to note that some genes that mediate Cd tolerance are not regulated at the transcript level by exposure to toxic metal(loid)s. AtABCC1 and AtABCC2, the recently identified vacuolar PC transporters, are not induced by As, but are constitutively expressed [9••]. It is thought that this strategy allows for rapid storage and detoxification of these and other toxic xenobiotic compounds in the vacuole [9••].

The signaling pathway responsible for Cd-induced gene expression remains largely unknown. However, it was recently shown that Cd can activate the mitogen-activated protein kinases MAPK3 and MPK6[66]. This is supported by previous studies showing that the MAPK pathway is important for reactive oxygen species (ROS) signaling [67–69]. Both ROS signaling and nitric oxide (NO) have been implicated as early signals in the Cd signal transduction pathway [70–71]. Thus, the question of how Cd and As cause rapid gene expression and which transcription factors mediate early Cd and As transcriptional regulation are open questions.

Concluding Remarks

Metal accumulation and homeostasis require the coordination of several processes working simultaneously to regulate uptake, long-distance transport and distribution of metals to different cells and tissues (Fig. 1). In recent research, key missing transporters mediating metal and arsenic tolerance, vacuolar accumulation and distribution in plants have been identified. Since plants and seeds represent the major source of metal intake for humans and livestock, an in-depth understanding of these processes could help to ensure the accumulation of *essential* nutrient metals and avoid the retention of toxic metals in the food supply. Furthermore, phytoremediation has received significant attention as an improved alternative to physical removal strategies to restore sites contaminated with metals and metalloids. The recently identified mechanisms for vacuolar detoxification and sequestration of cadmium and arsenic, together with the novel genes discovered in natural metal hyperaccumulator species offer new targets to engineer fast-growing high-biomass producing metal hyperaccumulator organisms. This will likely require the simultaneous increase of metabolic flux through the thiol synthesis pathway [56] together with metal(loid) and thiol-metal(loid) transporters to manipulate tolerance, accumulation and allocation of metals within the plant.

Highlights

1. The ABC transporters Abc2 (*S. pombe*), ABCC1 (*A. thaliana*), and ABCC2 (*A. thaliana*) have been identified as the long-sought vacuolar phytochelatin transporters.
2. The vacuolar ATPase HMA3 affects shoot-root partitioning of cadmium.
3. Cadmium in seeds is coordinated by thiol-containing compounds.
4. Heavy metals induce transcriptional responses that allow plants to preserve metal homeostasis.

5. The transcriptional regulators required for Cd- and As-induced gene expression remain unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Uptake, sequestration and long-distance cadmium and arsenic transport mechanisms in plants.

(a) Cadmium (Cd^{2+}) uptake, at the root level, is mediated by ZIP transporters (i.e. IRT1 transporter) and other divalent metal nutrient transporters. Arsenic, depending on the redox state [As(V) or As(III)], may be taken up by phosphate transporters [As(V)] [33] or nodulin 26-like proteins [As(III)] [4–5]. Once inside the cell As(V) is readily reduced to As(III) . Cd^{2+} and As(III) induce phytochelatin (PC) synthesis in the cytosol and PC-metal(loid) complexes are sequestered into vacuoles by ABCC transporters (ABCC1 and ABCC2 in Arabidopsis [9]). ABCC1 and ABCC2 can also transport GS-X conjugates into vacuoles

[16]. Cd^{2+} can also be sequestered into vacuoles by HMA3 and proton/cation exchange transporters (CAX-type transporters). Cadmium can also be released from the vacuole by NRAMP-type transporters [72–73].

(b) Loading of Cd^{2+} into the xylem for root-to-shoot transport of Cd^{2+} is mediated by the ATPases HMA2 and HMA4 [39,63]. Similar to root-uptake, NIP-like transporters mediate As(III) loading into the xylem while phosphate transporters mediate loading of As(V) [4–5].

(c) As(V) may also be reduced in leaves to As(III). Phytochelatins induced by Cd^{2+} or As(III) in leaves are transported into vacuoles by ABCC transporters. Similar to root vacuoles, Cd uptake is also mediated by CAX-type and MTP-type transporters [4–5]. Depending on the species, metal(loid)s may be accumulated in epidermal or mesophyll cells [4,74].

(d) If Cd^{2+} and As(III) are not fully sequestered in leaf vacuoles, they can reach phloem parenchyma and companion cells through plasmodesmata (symplastic transport). Due to the high permeability of companion cells and sieve element plasmodesmata, GSH, As(III)- or Cd-induced PCs synthesized in companion cells may enter the phloem stream symplastically and be transported from source-to-sink (i.e. to young leaves, seeds and roots) [35].

(e) EXAFS analysis in the Cd hyperaccumulator *Thlaspi praecox* showed that 60% of Cd in seeds is complexed with thiol-containing compounds [42] and a similar mechanism was also proposed for arsenic accumulation in rice grains [75].

Figure 2.

Maximum likelihood phylogenetic tree of AtABCC2 and ABC-type transporters showing the highest sequence similarity to AtABCC2 in higher plants and selected fungi and animals. ABC transporters fall into two main clades characteristic for plants and animal or fungi [76–78]. The only functionally characterized vacuolar phytochelatin transporters shown are *A. thaliana* ABCC2 and *S. pombe* Abc2 [8–9]. A detailed explanation for gene annotations is provided in Supplementary Table 1. Note that the following organisms lack phytochelatin: *P. patens*, *S. cerevisiae* and *D. melanogaster*. The tree was constructed using phym1 with 100 bootstrap replicates on a rascal curated mafft alignment of the shown proteins [76–78]. Nodes labeled with two asterisks have a 100% bootstrap support while those with one asterisk have a support in the range of 90% to 100%. The scale bar indicates 0.2 substitutions per site.