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Detoxification and Accumulation of Cadmium and Arsenic in Plants: Implications for Phytoremediation and Limiting Accumulation in Foods

A dissertation submitted in partial satisfaction of the requirements
for the degree of Doctor of Philosophy.

in

Biology

by

Timothy O. Jobe

Committee in Charge:

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2013

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2013

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Appendix 1, in full, is a reprint of the material as it appears in The Plant Journal 2012. Jobe, T. O., Sung, D.-Y., Akmakjian, G., Pham, A., Komives, E. A., Mendoza-Cózatl, D. G. and Schroeder, J. I. (2012), Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals glutathione-deficient γ-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation. The dissertation author was the primary author of this paper.

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Jobe, T. O., Sung, D.-Y., Akmakjian, G., Pham, A., Komives, E. A., Mendoza-Cózatl, D. G. and Schroeder, J. I. (2012), Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals glutathione-deficient γ-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation. *The Plant Journal,* 70, 783-795.

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ABSTRACT OF THE DISSERTATION

Detoxification and Accumulation of Cadmium and Arsenic in Plants: Implications for Phytoremediation and Limiting Accumulation in Foods

by

Timothy O. Jobe

Doctor of Philosophy. in Biology

University of California, San Diego, 2013

Professor Julian I. Schroeder, Chair

Many of the metals and metalloids commonly used by our modern society are extremely toxic and can pose a significant health risk if consumed. However, unlike animals, some plants are often extremely tolerant to the toxic effects of these metals and can accumulate large amounts in various tissues. Because some plants can bioaccumulate toxic metals, a number of bioremediation strategies using plants have been proposed. However, accumulation of toxic metals in agronomic crops is not desirable. In fact, interest is growing within the plant breeding community to reduce the accumulation of toxic metals in key crops. Whether we are interested in increasing accumulation for remediation, or reducing accumulation for human consumption, understanding the

molecular and genetic mechanisms underlying toxic metal sensing, uptake, detoxification, and storage are paramount for success. This dissertation outlines the systematic approaches we have taken to understand many of these processes. In the first part of this dissertation, the cloning and screening of cadmium/arsenic-inducible promoter elements using a genome-wide yeast one-hybrid approach along with microarray analyses of known mutants are presented. This is a continuation of the work presented in Appendix 1, which focuses on developing a cadmium/arsenic-inducible reporter line and screening, identifying, and characterizing new mutants in the glutathione biosynthesis pathway. Appendix 2 is a review article highlighting recent advances in the field of toxic metal tolerance and presents key gaps in our knowledge.

Chapter 1:

Genome-wide yeast one-hybrid to identify transcriptional regulators of sulfate assimilation genes

1.1. Introduction

Cadmium, arsenic, lead, and mercury are toxic metal(loid)s that have no known role in animal or plant nutrition and are considered detrimental to human health and the environment. These substances are among the top 7 contaminants listed on the EPA Superfund's list of priority hazardous substances, and can be found at high levels in soils and waters throughout the country. Widespread contamination from anthropogenic inputs is a growing problem. Metals are widely used in industrial processes and equipment, including battery production, electronics, paints, fertilizers and fuel production [1,2]. Industrialization has drastically increased emissions of toxic metal(loid)s into the environment, which is a major concern for people living near industrial areas where toxic metal(loid) emissions are highest [3]. Metal contamination has become a serious worldwide environmental health issue due to two centuries of intense industrial activity combined with inappropriate waste disposal [2,4,5]. Many diseases and disorders have been linked to high levels of toxic metal(loid) exposure, including high rates of lung cancer in workers from cadmium recycling and recovery facilities[6 7]. The toxic effects of heavy metals have also been linked to hypertension, myocardial infarction, and diminished lung function [8].

Plants and seeds are the main dietary source of many essential metals, including zinc, iron, manganese and copper, not only for humans but also for livestock [9-11]. Plant-based products are also the main entry point for toxic elements (cadmium, lead, arsenic, and mercury) into the food chain, and many cases of heavy metal poisoning have been attributed to widespread consumption of contaminated products [12-14]. Understanding the molecular mechanisms underlying plant uptake, transport and accumulation of both

essential and non-essential metals will have two major impacts on human health. First, it will enhance the nutritional value and safety of plant products by enhancing the accumulation of essential metals while avoiding the retention of toxic metals. Second, it will allow us to use plants to restore and remediate heavy metal contaminated sites, which is a preferred alternative to physical removal of metals. The identification of genes and molecular mechanisms that allow plants to take up, tolerate and accumulate toxic metals will accelerate the engineering of plants for remediation purposes.

1.1.1 Metal uptake and mobilization

The distribution of metals within a plant is a dynamic process that can be divided into the following processes: (i) root uptake and intercellular mobilization, (ii) xylem-loading/unloading and (iii) phloem-loading/unloading. In Arabidopsis, Fe, Cd, Mn and Zn are taken up from soil by IRT1, a member of the ZIP (Zinc/Iron regulated transporter Proteins) family of transporters [15-17], while arsenic has been shown to be taken up by inorganic phosphate transporters. Once inside the cell, metals can be sequestered into different cellular compartments or mobilized through the root for xylem loading and root-to-shoot transport [18,19]. Cadmium uptake by IRT1 and xylem loading mediated by HMA2 and HMA4 have been extensively studied[15,20-22]. However, phloem transport and seed loading have been far less studied, perhaps due to the technical difficulties associated with phloem sampling and sap modification. Phloem plays a key role in delivering compounds to developing seeds where xylem-mediated transport is negligible due to the limited transpiration rate within reproductive tissues[23]. Phloem is a plant tissue composed of two highly specialized low-abundance cells called companion cells and sieve elements

[23]. Companion cells transfer molecules into sieve elements for long-distance transport between mature leaves, younger leaves, roots and seeds. Thus, transporters expressed in companion cells are critical proteins regulating the long-distance movement of molecules, including toxic metals. Despite their importance, the identity and abundance of phloem-specific metal transporters as well as their regulation during plant development is largely unknown.

The Arabidopsis genome is estimated to have approximately 25,500 open reading frames (ORFs or potential genes), of which 7% (approx. 1700 genes) are predicted to encode transporters. Based on this large number of transporters and the potential for overlapping function between members of the same gene families, a forward genetic screen designed to identify genes mediating the mobilization of molecules into seeds would be time consuming and inefficient. Therefore, alternate approaches must be developed to identify and characterize the transporters involved in mobilizing metals throughout the plant.

1.1.2 Toxic metal accumulation and storage in plants

Phytochelatins are glutathione-derived peptides synthesized in the cytosol upon exposure to Cd, As, Zn, Hg or Cu [24-26]. After being synthesized, they rapidly form PC-metal complexes that are transported into vacuoles, removing these toxic elements from the cytosol [25-28]. More than 15 years ago, research suggested that vacuolar uptake of PC-metal complexes was mediated by ATP-binding cassette transporters (ABC transporters) [29-31]. However, attempts to identify vacuolar PC transporters in plants were unsuccessful

[27]. By conducting a systematic analysis of the ABC transporter family in the fission yeast *Schizosaccharomyces pombe*, we were able to identify Abc2 as a novel vacuolar PC transporter [32]. Our results indicated that *S. pombe* has two independent mechanisms for vacuolar sequestration of PC-Cd complexes, one mediated by Hmt1, a half-size ABC transporter [30,32,33] and a different mechanism mediated by Abc2, a full-size ABC transporter[32]. Notably, plants do not have Hmt1 homologues but they do have homologues of Abc2, which are the ABCC family of ABC transporters [34].

Furthermore, Arabidopsis mutants carrying T-DNA insertions in genes displaying a high degree of similarity with *S. pombe* Abc2 were subsequently identified that led to the identification of ABCC1 and ABCC2 as the long-sought plant vacuolar PC transporters [23,35]. Both, ABCC1 and ABCC2 are able to mediate the uptake of PCs in vacuolar preparations obtained from yeast expressing either ABCC1 or ABCC2 [35]. The single insertion mutants *abcc1* and *abcc2* are not sensitive to either Cd or As but the double mutant *abcc1 abcc2* is both Cd, Hg and As hypersensitive [35,36]. *ABCC1* and *ABCC2* are expressed approximately 3-fold higher in roots compared to shoots [23,37] and in the Arabidopsis ecotype Col-0, roots are the main sink for Cd storage [38,39].

Phytochelatins have long been considered part of an intracellular mechanism for Cd detoxification [24,25]. However, recent evidence suggests that PCs also play a key role in mobilizing cadmium from leaves to roots [38,40]. Shoot-specific expression of PC-synthase in a PC-deficient mutant showed that despite being synthesized in leaves, PCs were preferentially accumulated in roots[38]. Transport of molecules from leaves to roots occurs exclusively through the phloem and direct analysis of phloem and xylem sap by mass spectrometry demonstrated that PCs were more abundant in the phloem sap compared to

the xylem sap and in sufficient quantities to chelate the Cd found in phloem sap [40]. These results led to a model were Cd is removed from leaves to protect photosynthesis, which is extremely sensitive to Cd [23]. Other metal ligand molecules found in phloem sap are nicotianamine and GSH [40-42]. Nicotianamine has been shown to form complexes with Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} [43], while GSH and PCs have orders of magnitude higher affinities for the heavy metal(loid)s Cd2+, Zn2+, Hg2+ and As3+ {Mendoza-Cozatl, 2008 #523. In fact, extended X-ray absorption fine structure (EXAFS) analysis of seeds shows that 60% of Cd is coordinated with thiol-containing ligands [23].

The mechanisms by which GSH, PCs and toxic metals are loaded into the phloem are not known. The identification of phloem transporters will allow us to address these questions and advance our understanding of how molecules are mobilized between leaves and roots and into seeds.

1.1.3 Phytoremediation

Trace metals, such as iron, zinc, manganese and copper are essential micronutrients to all organisms and function as co-factors in a variety of enzymes and proteins [28,43]. It has been estimated that one-third of all proteins require one of these metals for proper folding and activity. Trace metals are highly reactive and their intracellular concentration must be tightly regulated to prevent toxicity. Other metals, such as cadmium (Cd), lead, chromium, mercury and the metalloid arsenic are biologically non-essential but, because of their chemical similarity, can enter plants using the same transporters used for essential metals [23,26]. Inside the cells, non-essential metals impair metabolism by displacing and interfering with the function of essential metals.

Non-essential metals are toxic to plants at any level; however, essential metals can also be toxic if they accumulate to high levels.

Traditional methods of remediating metal contaminated soil and water include excavation, transport, and reburial of contaminated soil and evaporation, filtration or electro-chemical removal from contaminated waters. These methods are both labor and energy intensive making them cost ineffective. Because plants are sessile and have little control over the soil or water they must survive in, they have developed unique strategies to cope with metal(loid) toxicity. This makes them well adapted for use in the bioremediation of highly contaminated sites (referred to as phytoremediation).

Cadmium is an important pollutant due to its relatively high solubility and toxicity. Cadmium has no distinct function in human health and is extremely toxic at low concentrations. The main oxidation state of cadmium is +2, which means that it can interfere with calcium, copper, iron, magnesium, and manganese containing enzymes by displacing these elements and competing with transport [44]. While cadmium cannot undergo Fenton-type reactions, it is highly reactive with sulfhydryl groups and is thought to cause lipid oxidation.

Arsenic is an extremely toxic metalloid, and arsenic pollution has been recognized as an environmental problem worldwide. Arsenate (+5) is a chemical analogue of phosphate and can disrupt phosphate metabolism in plants, while arsenite (+3) is highly reactive with the sulfhydryl groups of enzymes and proteins, causing oxidation of proteins, inhibition of cellular function and cell death [45]. Because cadmium and arsenic are both highly reactive with sulfhydryl groups, they are detoxified by similar mechanisms in plants.

Therefore, it is important to investigate the mechanisms by which plants take up and detoxify metal(loid)s. Research over the past few decades indicates that uptake and subsequent accumulation of toxicants in the aerial portions of plant tissues could provide a cost effective approach for cadmium and arsenic removal and remediation.

1.1.4 Cadmium and Arsenic Detoxification in Plants

The mechanisms underlying cadmium and arsenic detoxification are biochemically well established. Exposure to cadmium and arsenic enhances the expression of sulfate assimilation genes for detoxification [46,47]. Sulfur is a macronutrient in plants and is available primarily in the form of sulfate (SO_4^{2-}) present in soil [48]. Sulfate is actively transported into roots and then distributed throughout the plant. The high affinity sulfate transporter, SULTR1;2 is induced by both arsenic and cadmium [47]. Once sulfate enters the root, it is reduced in a series of ATP-dependent reactions and incorporated into the amino acid cysteine (Cys). Cysteine can then be converted to methionine, glutathione (GSH), and other sulfur-containing metabolites. GSH, the most abundant thiol molecule in plant cells, is synthesized in two ATP-dependent steps catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSHS) [49]. Upon toxic metal exposure, plants also produce thiol compounds called phytochelatins, which are polymers of GSH. Phytochelatins (are synthesized from GSH by the enzyme phytochelatin synthase and have a general structure of (γ-glutamylcysteine)$_n$Gly (n=2-11) [50 51 52 53]. PCs have a very high affinity for cadmium and arsenic, which allow PCs to quickly chelate and sequester them in the vacuole [54]. PC

production can be induced by a wide range of ions, including Ag^{+}, $As^{(V)}$, Cd^{2+}, Cu^{+}, Hg^{2+}, Cu^{+}, Hg^{2+}, and Pb^{2+} , with Cd^{2+} being the most potent[54-56].

Unfortunately, our understanding of the cellular signaling underlying heavy metal uptake, transport, and accumulation in plants remains incomplete. The complexity of signal transduction in higher plants is a result of spatial and temporal signal separation, cross talk between signaling networks, and extensive genetic redundancy. As a result, classical forward genetic screens have proven ineffective at elucidating the signaling cascades responsible for plant responses to many different external stimuli.

1.1.5 Cadmium and Arsenic Signaling in Plants

While the biochemical mechanisms for metal(loid) detoxification are well understood, the transcriptional regulation is still largely unknown. Recently, point mutants in a gene called s*ulfur limitation1 (SLIM1)* were identified that play an important role in the regulation of the sulfate assimilation pathway under sulfur limiting conditions (-S). SLIM1 is a central transcriptional regulator that is reported to activate the *SULTR1;2* high affinity sulfate transporter in *Arabidopsis* under -S conditions [57]. The transcript levels of *SULTR1;2* are nearly abolished under –S conditions in the s*lim1-1* and *slim1-2* mutants [57]. Metabolite accumulation was also affected in the *SLIM1* mutants, where *slim1-1* and *slim1-2* show a significant decrease in GSH levels compared to wild type when grown on low sulfur media [57]. These findings indicate the importance of SLIM1 in sulfur metabolism.

The transcriptional regulation of heavy metal(loid)-induced gene expression remains largely unknown in plants. While SLIM1 is a central regulator of the sulfate

assimilation pathway, it is unclear if SLIM1 plays a role in activation of sulfate assimilation during metal(loid) detoxification. The complex regulation of sulfate assimilation and the unknown role of SLIM1 in metal(loid) detoxification raises the question whether SLIM1 acts together with other yet unknown transcription factors to be part of a large regulatory network . However, the large number of transcription factor families and the potential partially unequal redundancy of certain family members represent a challenge. Recently, a small library became available which addresses genetic redundancy in Arabidopsis using artificial microRNAs[58]. Targeting multiple homologous transcription factors allowed for screening of large groups of genes, while reducing or eliminating functional overlap[58]. Unfortunately, the initial screen was unable to narrow down potential transcription factors involved in metal(loid) detoxification. A larger library of amiRNA lines is now available that may allow us to gain a deeper understanding of specific transcriptional activators and repressors involved in metal detoxification[58].

In this dissertation, we present work aimed at uncovering the genes and mechanisms underlying the cadmium and arsenic transcriptional responses using a variety of approaches.

1.2. Results

Genome-wide Yeast One-Hybrid

To identifying transcription factors that mediate Cd/As-induced gene expression in plants, we performed genome wide yeast one-hybrid analyses on the promoter fragments of four Cd/As-induced genes, specifically the *SULTR1;2*, *APR2*, *γ-ECS*, and

GS promoters. Our goal was to identify the unknown master transcriptional regulators and repressors that control the promoters of multiple Cd/As-induced genes. We cloned the 2.1 kb promoter region previously characterized for SULTR1;2 as well as a 2.1 kb promoter fragment from APR2. The promoter fragments were shorter for g-ECS and GS due to the presence of upstream genes. The g-ECS promoter was approximately 1.4 kb in length, while the GS promoter was 800 bp.

We used these promoter fragments to screen approximately 2,100 Arabidopsis transcription factors for DNA-protein interactions in a yeast one-hybrid assay. The GS, γ-ECS, and APR2 promoters were screened using a previously published transformation based protocol[59]. The SULTR1;2 promoter was screened using a mating-based protocol. Because we had little experience with the new protocol, we screened the SULTR1;2 promoter 3 times to determine the level of reproducibility of this method. Figure 1 shows a heat map of the β-gal values determined from each of the 6 screens we performed. In general, the mating-based protocol identified more putative DNA-protein interactions; however, there was a low degree of reproducibility between the 3 replicate experiments. For the 3 transformation-based screens, a smaller number of putative interactions were identified.

Interestingly, a number of transcription factors were identified in more than one screen. For simplicity, we averaged the β-gal values between the 3 SULTR1;2 screens and compared with the β-gal values obtained from the remaining screens. We compared the results in two different ways. First, we considered interactions having z-scores ≥ 2.0, which is traditionally used in yeast one-hybrid analyses. Secondly, we performed outlier detection analysis using an R script. Figure 2 shows Venn diagrams indicating the

number of interactions identified and the overlap between the screens using both the z-score method (Figure.. 2A) and the outlier detection method (Figure.. 2B). In general, the outlier detection algorithm identified more putative interactions and more overlapping interactions between the different promoters than the z-score method. However, in both cases the total number of putative interactions was unexpectedly high.

To look more closely at the interactions identified by these screens, we decided to focus on the three interactions from each screen with the highest total β-gal values. Table 1 shows the 3 interactions with the highest β-gal values from each of the 4 yeast one-hybrid screens along with the number of putative interactions with a β-gal value of 2.0 or greater. We obtained T-DNA insertion lines in these genes and performed root elongation assays on the individual mutant lines to determine their sensitivity to cadmium. Only one mutant, Salk_094437C, was identified as having a cadmium-dependent phenotype. We obtained an additional T-DNA disruption mutant in the At2G46270 (ANAC102) gene. Our initial screen of these T-DNA mutants revealed that both independent alleles of ANAC102 as showing Cd tolerance (*anac102-1*, Salk_030702C; *anac102-2*, Salk_094437C Figure.. 3). No differences in growth were observed in the absence of Cd.

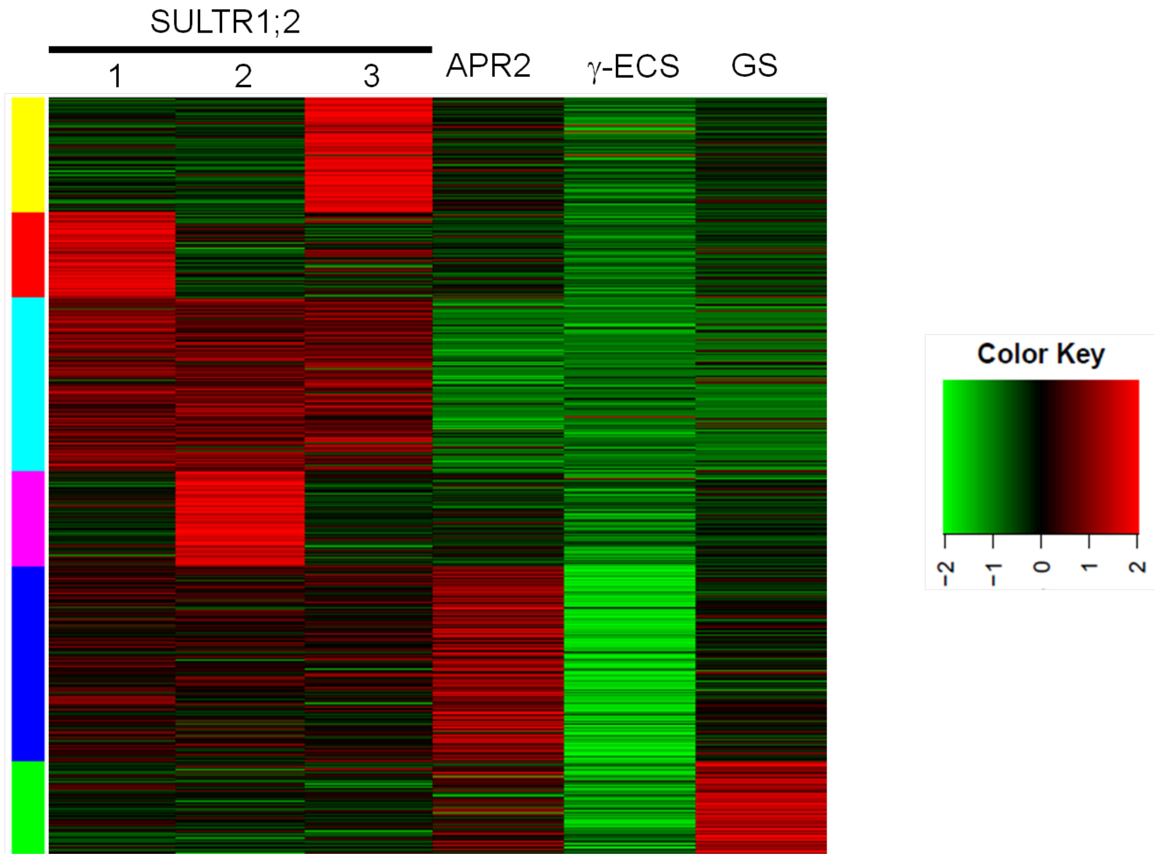

Figure.. 1: Heat map of the 6 yeast one-hybrid experiments performed. Three independent replicate experiments were performed on the SULTR1;2 promoter (represented as 1, 2, and 3). The APR2, g-ECS and GS promoters were screened using a transformation based protocol, while the SULTR1;2 promoter was screened using a mate-based protocol.

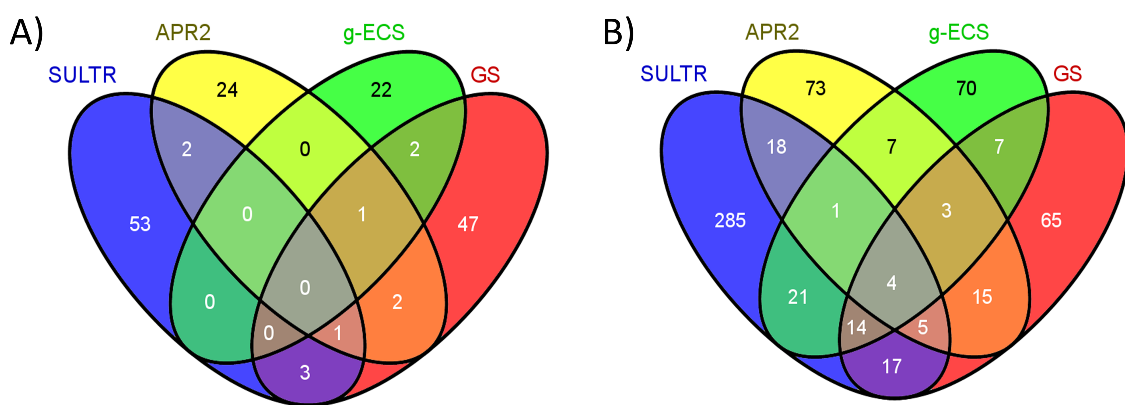

Figure.. 2: Venn diagram of the yeast one-hybrid results. A) The results were filtered by z-score using a score of 2.0 or greater as indicating a putative DNA-protein interaction. B) The results were filtered using an outlier detection program.

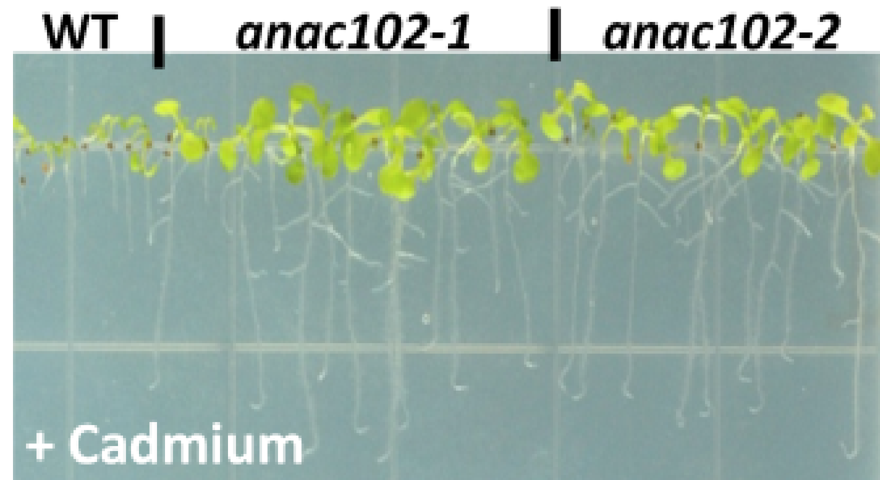

Figure.. 3: Root elongation assay of *anac102-1* and *anac102-2* mutants. Two independent T-DNA disruption mutant alleles in a NAC transcription factor At5G63790 (ANAC102) show enhanced Cd tolerance in Cd-sensitive root growth assays. Wild type (WT) and two independent T-DNA alleles, *anac102-1* (Salk_030702C) and *anac102-2* (Salk_094437C), were grown with Cd for 7 days. Seedling growth of the mutant alleles was drastically longer than WT.

Table 1: Top three ranked transcription factors for each promoter

Promoter	Gene ID	Gene Name	Maximum (z-score >=2)
APR2	AT1G49720	ABF1	
APR2	AT5G63790	NAC102	
APR2	AT5G65210	TGA1, a redox-controlled regulator	37
γ-ECS	AT3G61890	HB12	
γ-ECS	AT5G15800	SEPALLATA1 (SEP1)	32
γ-ECS	AT5G41570	WRKY24	
GS	AT1G36060	DREB	
GS	AT2G40470	LBD15	65
GS	AT4G04890	PDF2	
SULTR1;2	AT2G01370	storekeeper related	
SULTR1;2	AT2G41070	EEL	
SULTR1;2	AT5G62920	ARR6	62

1.3. Discussion

The genome-wide yeast one-hybrid screen identified numerous putative DNA-protein interactions. The three SULTR1;2 promoter screens showed a low level of reproducibility (see Figure.. 1). However, there was significant overlap between several of the clusters. Interestingly, the remaining three screens showed far fewer interactions than the SULTR1;2 promoter screens. Our hypothesis that a limited number of transcriptional regulators are responsible for the co-regulation of these four cadmium-inducible genes was partially supported by the level of overlap identified between the four promoter screens (Figure.. 2). Unfortunately, the large number of putative overlapping interactions makes it difficult to determine which individual transcription factor or group of transcription factors may play a significant role in mediating early transcriptional responses to cadmium and arsenic.

To further narrow down the most promising candidates, we also ranked the hits from the yeast one-hybrid screens and focused on the three hits with the highest Z-scores (Table 1). Initial root-elongation screens using mutants in these 12 genes identified a single mutant in the ANAC102 gene having a long-root phenotype on cadmium (Figure.. 3). Further characterization showed that two different mutant alleles, *anac102-1* and *anac102-2* both had similar root elongation phenotypes on cadmium. Unfortunately, both of these mutants also exhibited long-roots when grown without cadmium, which suggests the phenotype is not cadmium or arsenic specific. These results suggest that additional screening of higher order mutants may be required to identify the genes that specifically mediate cadmium and arsenic responses in plants.

1.4. Materials and Methods

Ecotypes

The wild type *A. thaliana* ecotype used was Columbia-0 (Col).

Plant Growth Conditions

Seeds were sterilized and plated on plates containing one-quarter strength Murashige and Skoog (MS) standard medium (Sigma M5519), 1 mM MES, 1% phytoagar (Duchefa), and the pH adjusted to 5.6 with 1.0 M KOH. Sterilized nylon mesh with a 200 μm pore size (Spectrum Labs, TX) was placed on the surface of the media prior to sowing the seeds. The seeds were then acclimated with cold treatment at 4 ^{o}C for 48 hours, and grown under growth room conditions for five days (300μmol.m$_{-2}$.s$_{-1}$, 70% Hr, 16h light 21°C/8h dark 18°C). Seedlings were then transferred to quarter strength

MS, 1 mM MES, 1% agar plates containing 20 µM $CdCl_2$.

For hydroponic cultures, plants were grown on ¼ MS plates for 6 weeks under growth chamber conditions as described above. Seedlings were then transferred from ¼ MS plates to polystyrene rafts floating on hydroponics solution. The composition of the hydroponics solution is as follows: KNO_3 0.5mM, $Ca(NO_3)_2$ 0.25mM, $MgSO_4$ 1mM, KH_2PO_4 1mM, Fe(III)-Na-EDTA 100µM, H_3BO_3 50µM, $MnCl_2$ 19µM, $CuSO_4$ 1µM, $ZnCl_2$ 10µM, MoO_4Na_2 0.02µM, pH 5.8. The nutrient solutions were replaced every seven days.

Root Growth Assays

For root growth analysis Arabidopsis seedlings were grown horizontally on one-quarter-strength Murashige and Skoog (MS) basal medium (Sigma Aldrich), 1 mM MES, 1% agar for a period of four days. Seedlings and nylon mesh were then transferred to MS plates supplemented with 100 µM CdCl2 for an additional four days. Seedlings used for shoot growth analyses were grown horizontally under 16/8-h day/night period at 23°C.

To measure metal accumulation, plants were grown under hydroponic conditions in liquid media until bolting stage (approximately 4 weeks). Shoots were rinsed 3 times in deionized water. For ICP-OES analyses of root tissue, roots were rinsed in deionized water, washed in 100 mL of 100 mM CaCl2 on an orbital shaker (Bellco Glass, Inc., NJ) for 5 minutes at approximately 135 rpm (speed setting #4), and then washed in 100 mL of deionized water on the orbital shaker (speed setting #4) for an additional 3 minutes. Shoot tissues were separated immediately below the cotyledons and above the

hydroponic sponge. Root tissues were separated below the hydroponic sponge, approximately 3 cm below cotyledons. Both shoot and root tissues were dried at 60oC overnight. The dry weight was recorded, and then the tissues were digested in 70% trace metal grade nitric acid (Fisher Scientific) overnight. Samples were then boiled for 30 minutes to ensure complete digestion and diluted to a final concentration of 5% nitric acid with deionized water.

Metal Determination Using ICP-OES

To measure the metal accumulation of plate-grown plants, Arabidopsis seedlings were grown in minimal media (Lee et al., 2003) for 4 days then exposed to 100 μM metal(loid) by transferring seedlings with nylon mesh to minimal media with metal(loid). Plants were allowed to continue growing in arsenic-containing medium for 6, 12, 24 and 48 h. The plants were then washed briefly with distilled water twice. The plant seedlings were dried in a drying oven at 60°C overnight, the dry weight was measured, and the dry material was digested by boiling in concentrated nitric acid (Trace Metal grade; Sigma-Aldrich; http://www.sigmaaldrich.com/). These digests were then diluted with deionized water and the metal content of the digested samples was analyzed with a Varian Vista Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). Total concentrations of metals were normalized based on the dry weight of the plant samples.

Plants used for Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) and Fluorescence HPLC analysis were grown under hydroponic conditions. Plants were grown under hydroponic conditions at 24°C under a 16/8-h day/night period and

hydroponic media were replaced every 3-4 days. After reaching the bolting stage (approximately 4 week old plants) the hydroponic media was replaced with the same media to which 20 mM $CdCl_2$ was added for a period of four days for cadmium accumulation analyses, whereas the hydroponic media containing 20 μM $CdCl_2$ was added for a period of two days for induction of PCs. Shoot and root tissues were rinsed three times in deionized water for fluorescence HPLC coupled to mass spectrometry analyses. Thiols, including PCs were analyzed using the method described by Jobe et al. (2012).

To measure metal accumulation, plants were grown under hydroponic conditions in hydroponic media (12.5 mM KNO3, 6.25 mM KH2PO4, 5 mM MgSO4, 5 mM Ca(NO3)2, 125 mM Fe-EDTA, 3.5 mM H3BO3, 1.1 mM MnCl2, 100 mM ZnSO4, 12.5 mM NaMoO4, 500 mM NaCl, 900 mM CoCl2) until the bolting stage. Cadmium treated plants grown under hydroponic conditions were first washed and then separated into root and shoot tissues. Shoot tissues were separated immediately below the cotyledons and above the hydroponic sponge. Root tissues were separated below the hydroponic sponge, approximately 3 cm below the cotyledons. Shoots were rinsed three times in deionized water. For ICP-OES analyses of root tissue, roots were rinsed in deionized water, washed in 100 mL of 100 mM CaCl2 on an orbital shaker (Bellco Glass, Inc., NJ) for 5 minutes at approximately 135 rpm (speed setting #4), and then washed in 100 mL of deionized water on the orbital shaker (speed setting #4) for an additional three minutes. Both shoot and root tissues were dried at 60oC overnight. The dry weight was recorded, and then the tissues were digested in 70% trace metal grade nitric acid (Fisher Scientific)

overnight. Samples were then boiled for 30 minutes to ensure complete digestion and diluted to a final concentration of 5% nitric acid with deionized water.

Plant Transformation

Arabidopsis thaliana ecotype Col-0 was transformed using the floral dip method (Clough and Bent, 1998). The helper plasmid, pSoup, was used for the pGreenII-carrying strains (Hellens et al., 2000). Hygromycin selection of transformants was performed in both the T1 and T2 generations.

HPLC-MS analyses of thiol peptides

Seeds were sown on plates having sterile nylon mesh with a 200 µm pore size (Spectrum Labs, http://www.sprectrumlabs.com) on the surface of the media. Seedlings were grown under standard growth conditions for 14 days. Seedlings were then transferred to plates containing either control ¼ MS media or ¼ MS media supplemented with 100 µM $CdCl_2$ for 48 hours. Seedlings were harvested after 48 hours of treatment and immediately flash frozen in liquid nitrogen to minimize the oxidation of thiol compounds. Thiol-containing compounds (cysteine, γ-EC, GSH, and PCs) were extracted, and reduced thiols were derivatized and quantified by fluorescence HPLC-MS as described by Mendoza-Cozatl (2008). Thiol measurements were quantified using the XCALIBUR software package (Version 1.3, Thermo Scientific, http: //www.thermoscientific .com). Thiol standards of glutathione, cysteine and γ-EC were purchased from Sigma-Aldrich. All reported thiol quantities are means of three

biologically independent samples, and error bars indicate the standard error of the mean (SEM).

Total RNA isolation

Seedlings were grown under standard growth conditions for 14 days. Before sowing seeds onto plates, sterilized nylon mesh with a 200 µm pore size (Spectrum Labs, http://www.sprectrumlabs.com) was placed on the surface of the media. The seedlings were grown on nylon mesh for 14 days. The seedlings were then harvested and plant tissue was immediately flash frozen into liquid nitrogen for RNA extraction. Total RNA was extracted by using a commercial RNA extraction kit (RNeasy Plant RNA Isolation Kit; Qiagen, http://qiagen.com). A total of 5 µg of RNA was treated with DNase 1 to remove DNA contamination (Invitrogen, http://www. invitrogen.com).

cDNA synthesis

1 µg of the DNase1 treated total RNA was reverse-transcribed with a *Not*I-d$(T)_{18}$ primer using a First Strand cDNA kit (GE Healthcare, http://www.gehealthcare.com) following the manufacturer’s instructions.

RT-PCR analysis

Reverse-transcribed cDNA was used for PCR. The following PCR conditions were used: 1) initial denaturation at 95˚C for 5 min, 2) DNA denaturation for at 95˚C for 15 sec, 3) primer annealing at 52˚C for 15 sec, 4) extension at 72˚C for 1 min, and 5) a final extension at 72˚C for 5 min. Steps 2-4 were repeated for 25-30 cycles. Elongation

factor-1α (EFF-1α) expression was used as a loading control. Table Z contains a list of all primers used in this study.

Quantitative RT-PCR analysis

PCR mixtures were prepared using SYBR® Green JumpStart™ Taq ReadyMix™ for Quantitative PCR (Sigma-Aldrich). The 384-well plates (BIO-RAD) were loaded with 5 µl of SYBR® Green JumpStart™ Taq ReadyMix™, 0.5 µl of cDNA, 2.5 µl of MQ water, and 1 µl (1.0 µM) of each primer for a total volume of 10 µl per well. Before starting the PCR, a short centrifugation step of 1 minute at 1,000 rpm was performed to ensure the mixture was at the bottom of each well. Reverse-transcribed cDNA was used for qRT-PCR on the CFX384 Touch™ Real-Time PCR Detection System (BIO-RAD) with the following conditions: 95˚C for 3 min followed by 40 cycles of 10 sec at 95˚C, 10 sec at 60˚C, and 15 sec at 72˚C. The products were analyzed with a melting-curve of 95˚C for 1 min, 60˚C for 2 min, and 50˚C for 2 sec, followed by a temperature cycle from 50˚C to 85˚C (0.5˚C/sec) and continuous fluorescence reading. Table 4 contains a list of all primers used in this study. Calculations were performed using the comparative C_T method (Schmittgen et al., 2008) and normalized to *EF1-α* expression.

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Appendix 1:

Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals glutathione-deficient g-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation

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Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals glutathione-deficient γ-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation

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SUMMARY

Plants exposed to heavy metals rapidly induce changes in gene expression that activate and enhance detoxification mechanisms, including toxic-metal chelation and the scavenging of reactive oxygen species. However, the mechanisms mediating toxic heavy metal-induced gene expression remain largely unknown. To genetically elucidate cadmium-specific transcriptional responses in Arabidopsis, we designed a genetic screen based on the activation of a cadmium-inducible reporter gene. Microarray studies identified a high-affinity sulfate transporter (*SULTR1;2*) among the most robust and rapid cadmium-inducible transcripts. The *SULTR1;2* promoter (2.2 kb) was fused with the firefly luciferase reporter gene to quantitatively report the transcriptional response of plants exposed to cadmium. Stably transformed luciferase reporter lines were ethyl methanesulfonate (EMS) mutagenized, and stable M_2 seedlings were screened for an abnormal luciferase response during exposure to cadmium. The screen identified non-allelic mutant lines that fell into one of three categories: (i) super response to cadmium (*SRC*) mutants; (ii) constitutive response to cadmium (*CRC*) mutants; or (iii) non-response and reduced response to cadmium (*NRC*) mutants. Two *nrc* mutants, *nrc1* and *nrc2*, were mapped, cloned and further characterized. The *nrc1* mutation was mapped to the γ-glutamylcysteine synthetase gene and the *nrc2* mutation was identified as the first viable recessive mutant allele in the glutathione synthetase gene. Moreover, genetic, HPLC mass spectrometry, and gene expression analysis of the *nrc1* and *nrc2* mutants, revealed that intracellular glutathione depletion alone would be insufficient to induce gene expression of sulfate uptake and assimilation mechanisms. Our results modify the glutathione-depletion driven model for sulfate assimilation gene induction during cadmium stress, and suggest that an enhanced oxidative state and depletion of upstream thiols, in addition to glutathione depletion, are necessary to induce the transcription of sulfate assimilation genes during early cadmium stress.

Keywords: glutathione biosynthesis, heavy metal, γ-glutamylcysteine synthetase, metabolite-based cloning, phytochelatins, *Arabidopsis thaliana*.

INTRODUCTION

Toxic metals such as lead, cadmium (Cd), mercury and the metalloid arsenic can accumulate in soils and water to levels that are detrimental to human and environmental health. Many human disorders have been attributed to the ingestion of heavy metals, including learning disabilities in children, dementia, impairment of bone metabolism and increased cancer rates (Tong *et al*., 2000; Allen *et al*., 2002; Aschner and Walker, 2002; Ohta *et al*., 2002; Yu *et al*., 2002; Waisberg *et al*., 2003; Heck *et al*., 2009; Satarug *et al*., 2010). Food crops are a major source of heavy metal intake in humans,

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which has prompted interest in understanding how plants take up, detoxify and retain heavy metals. In addition, plants hold the potential for the development of a cost-effective approach for the removal and remediation of heavy metal-laden soils and water through the use of metal-hyperaccumulating plants (phytoremediation) (Raskin *et al.*, 1994; Dushenkov *et al.*, 1995; Salt *et al.*, 1995, 1998; Clemens, 2006).

Metal trafficking, both within the cell and between different tissues, often requires the use of metal ligand molecules such as citrate, nicotianamine, glutathione (GSH) and phytochelatins (PCs) (Lee *et al.*, 1978; Grill *et al.*, 1985; Howden *et al.*, 1995; Kramer *et al.*, 2000; Sanchez-Fernandez *et al.*, 2001; Klein *et al.*, 2002; Richau *et al.*, 2009; Mendoza-Cozatl *et al.*, 2011). Glutathione is a crucial molecule required for the synthesis of PCs, which detoxify mercury, Cd and the metalloid arsenic. PCs are small glutathione polymers synthesized in the cytosol (Grill *et al.*, 1985; Clemens *et al.*, 1999, 2001; Ha *et al.*, 1999; Vatamaniuk *et al.*, 1999, 2001). PCs bind highly toxic heavy metals and metalloids, and transport them into the vacuoles by ABC transporters (Li *et al.*, 2004; Chen *et al.*, 2006; Mendoza-Cozatl *et al.*, 2010; Song *et al.*, 2010). Glutathione and PCs have been shown to undergo long-distance transport of Cd through the phloem, but the identities of these transporters remain unknown (Gong *et al.*, 2003; Chen *et al.*, 2006; Mendoza-Cozatl *et al.*, 2008). Thus, exposure to heavy metals can rapidly deplete glutathione levels and create an extremely high demand for glutathione.

At the transcriptional level, heavy metal exposure elicits a robust gene expression response in plants (Herbette *et al.*, 2006; Weber *et al.*, 2006). For instance, Cd exposure rapidly depletes cells of GSH, which in turn induces transcripts that encode sulfate uptake, sulfate assimilation and glutathione biosynthesis mechanisms (Lee and Leustek, 1999; Nocito *et al.*, 2006; Davidian and Kopriva, 2010). These findings have led to the development of a metabolite demand-driven model for the regulation of sulfate assimilation and glutathione biosynthesis in which heavy metal-induced GSH depletion induces gene expression (Vauclare *et al.*, 2002; Kopriva, 2006). However, the molecular mechanisms that trigger rapid changes in gene expression following heavy metal exposure in plants remain unknown.

To uncover the molecular and genetic mechanisms that mediate rapid Cd-induced gene expression in Arabidopsis, we have pursued Cd-induced microarray experiments and a forward genetic screen to identify mutants with altered responses to Cd exposure, using a Cd-inducible promoter driving the expression of the firefly luciferase gene. Unexpectedly, two of the mutants showing a dramatically decreased Cd response are impaired in steps upstream of GSH synthesis. HPLC-MS analyses of thiol compounds suggest that upstream thiols and an oxidative redox state functions in the induction of sulfate uptake genes during Cd exposure, and not during GSH depletion alone. Characterization of the transcriptional response to Cd in these mutants revealed a new level of regulation (hierarchical regulation) of sulfur assimilation signaling and glutathione biosynthesis in response to Cd exposure in plants.

RESULTS

Identification of a rapid cadmium-inducible promoter

To identify genes in Arabidopsis that are highly and rapidly induced by Cd, we performed oligonucleotide chip-microarray experiments (Affymetrix, ATH1) on 1-week-old Arabidopsis seedlings exposed to 200 μM $CdCl_2$ for 6 h (Table S1). Cd-inducible transcripts were identified (Table S1) and transcriptional activation following Cd exposure was confirmed for six strongly induced transcripts by RT-PCR (Figure 1a). Nine promoter-luciferase constructs containing 2.2-kb promoter fragments of the cadmium-inducible genes were introduced into Arabidopsis (Col-0), and stable T_3 homozygous seedlings were analyzed for cadmium-induced luminescence. Luciferase (LUC) reporter lines carrying the high-affinity sulfate transporter *SULTR1;2* promoter (*pSULTR1;2*) showed a quantitative and highly reproducible luciferase response to Cd, and one line (line A) was chosen for mutagenesis (Figure 1b). This *pSULTR1;2::LUC* reporter line will henceforth be referred to as the control reporter line or parental line.

pSULTR1;2::LUC is induced by cadmium, arsenate and copper, but not by exogenous reactive oxygen species

The dynamic response of 4 reporter lines was analyzed over a 12-h period following Cd exposure (Figure 1b,c). Luciferase activity was highest in roots, and the induction was evident after 1 h of Cd exposure, reaching a maximum after 3 h of exposure (Figure 1b) and decreasing steadily to half of the maximal induction after 12 h of Cd exposure (Figure 1c). To determine if the *SULTR1;2* promoter is induced broadly by metals or exogenous reactive oxygen species (ROS), luciferase induction was measured following exposure to arsenate, copper, aluminum, nickel, cobalt and the ROS-inducing agent paraquat (Figure 1d–i). Figure 1 shows that Cd (Figure 1c), arsenate (Figure 1d) and copper (Figure 1e) elicit a strong transcriptional response, whereas the remaining metals and paraquat showed limited or no induction during the 12-h exposure period. These results suggest that *SULTR1;2* is not broadly induced by oxidative stress and that the *SULTR1;2* induction line is a suitable parental line for a forward genetic screen to identify mutants with an impaired Cd-induced transcriptional response.

Seeds of the control reporter line were ethyl methanesulfonate (EMS) -mutagenized (approximately 6000 seeds), and 60 000 M_2 seedlings were screened for altered luciferase induction after 6 h of Cd exposure. Putative mutants were selected and the altered luciferase response was

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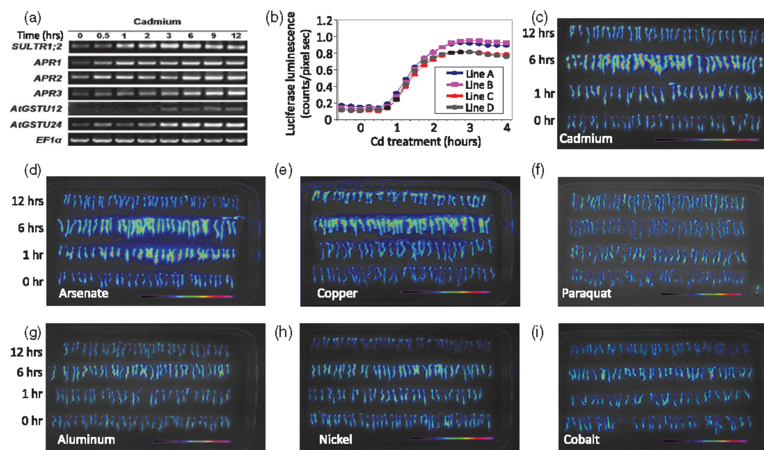

Figure 1. Development of a cadmium-inducible reporter line. (a) Genes identified by microarray analysis as being induced following 200 μM cadmium (Cd) treatment for 12 h were verified by RT-PCR. Tissue was collected every 30 min and RNA was extracted as described in the Experimental Procedures.
(b) Quantification of the luciferase activity of *pSULTR1;2::LUC* showing the dynamic response of four independent *pSULTR1;2::LUC* reporter lines over a 4-h time course. Measurements were taken in 15-min intervals.
(c–h) False color luminescence images after exposure to heavy metals, metalloids and reactive oxygen species (ROS)-generating compounds over a 12-h time course. Seedlings were exposed to 200 μM of the indicted substance and imaged as described in the Experimental Procedures. Specificity of the control reporter line response was determined by luminescence imaging after exposure to (c) cadmium, (d) arsenate, (e) copper, (f) paraquat, (g) aluminum, (h) nickel and (i) cobalt over a 12-h period.

confirmed in M_3 seedlings (Figure 2). Mutants were classified into one of three different groups: (i) *constitutive response without Cd* (*CRC*) mutants, showing a constitutive luciferase induction (Figure 2b) without being exposed to Cd; (ii) *super response to Cd* (*SRC*) mutants, which showed higher luciferase activity compared with the control reporter lines following Cd exposure (Figure 2c); (iii) *non-response or reduced response to Cd* (*NRC*) mutants, which failed to induce strong luciferase activity after Cd exposure (Figure 2d,e).

Glutathione-deficient mutants show reduced luciferase induction during Cd exposure

We focused on characterization of two recessive non-response mutants, designated *non-response to cadmium 1* and *2* (*nrc1* and *nrc2*), which are Cd sensitive and have short roots when grown in the presence of Cd. Figure 2(d,e) shows the luciferase phenotype of the *nrc1* and *nrc2* mutants. To validate the decreased luciferase response of the *nrc1* and *nrc2* mutants, RT-PCR analysis of the native *SULTR1;2* gene was performed in the control and in the *nrc1* and *nrc2* mutants. Figure 2(f,h) shows that the induction of the *SULTR1;2* transcript in *nrc1* was severely decreased compared with the control reporter line (Figure 2f), but only moderately decreased in the *nrc2* mutant (Figure 2h). The reduced size of *nrc2* seedlings probably contributed to the difference between the measured decreases in luciferase response in the *nrc2* mutant, with a moderate decrease in *Sultr1;2* transcript level in the *nrc2* mutant. Thus, the *nrc1* mutant is a strong non-response mutant, whereas the *nrc2* mutant, which retains some *Sultr1;2* induction, is more accurately described as a reduced response mutant. Root elongation experiments on plates containing 20 μM $CdCl_2$ showed that *nrc1* and *nrc2* seedlings are Cd hypersensitive (Figure 2g,i). Crosses between *nrc2* and *nrc1* showed that they are non-allelic.

The organic thiols cysteine, γ-glutamylcysteine (γ-EC) and GSH are known to be key metabolites required for the production of PCs that mediate Cd detoxification. Therefore, we analyzed the metabolic thiol profile of the *nrc1* and *nrc2* mutants by fluorescence HPLC coupled to a mass spectrometer (HPLC-MS) (Figure 3a–f). After exposure to 20 μM Cd for 48 h, GSH levels were decreased in both the *nrc1* (44.8 ± 2.31 nmol GSH per g fresh weight) and the *nrc2* (94.9 ± 8.22 nmol GSH per g fresh weight) mutants compared with parental controls (126.2 nmol GSH per g fresh weight; Figure 3a–c,f). Conversely, cysteine levels in the *nrc1* (66.6 ± 8.19 nmol Cys per g fresh weight) and *nrc2* (51.3 ± 8.93 nmol Cys per g fresh weight) mutants in the presence of Cd were elevated compared with parental controls (11.8 ± 0.71 nmol Cys per g fresh weight; Figure 3a–d). Interestingly, γ-EC levels were decreased

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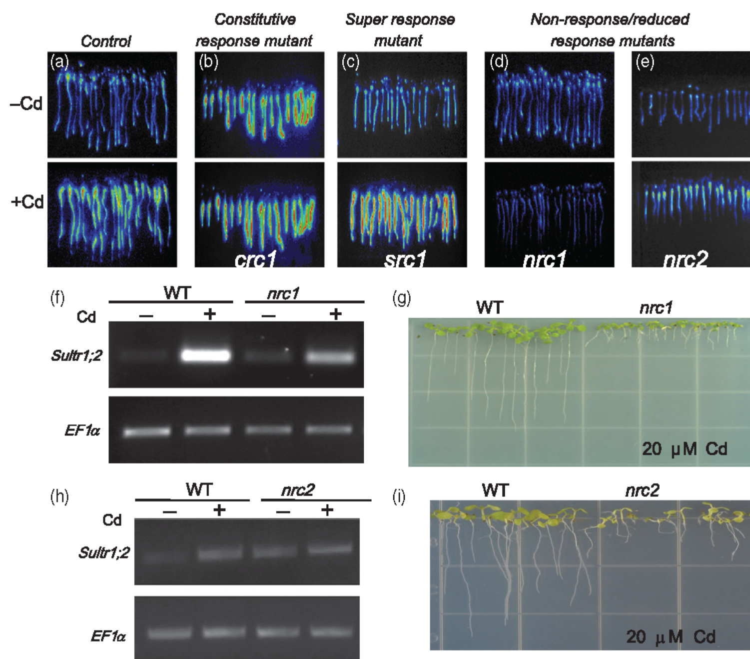

Figure 2. Ethyl methanesulfonate (EMS)-mutagenized *pSULTR1;2::LUC* seeds were screened for altered cadmium-induced luciferase activity responses: (a) mutants were classified into three different groups based on luciferase response, compared with the control reporter line; (b) *constitutive response to cadmium* or CRC mutants (*crc1*); (c) *super response to cadmium* or SRC mutants (*src1*); (d) *non-response to cadmium* or NRC mutants (*nrc1*, d; *nrc2*, e).
(f) RT-PCR of the native *SULTR1;2* expression using 35 PCR cycles confirmed the NR reporter phenotype of *nrc1*.
(g) *nrc1* shows a short root phenotype compared with control when grown on 20 μM cadmium for 9 days.
(h) RT-PCR of the native *SULTR1;2* transcript using 30 PCR cycles showed a reduced induction in the *nrc2* mutant. (i) *nrc2* also has a short-root phenotype compared with the control when grown on 20 μM Cd for 14 days.

following Cd exposure in the *nrc1* mutant (2.30 ± 0.40 nmol γ-EC per g fresh weight), and were elevated in the *nrc2* mutant (431.7 ± 72.7 nmol γ-EC per g fresh weight), compared with parental controls (8.57 ± 0.25 nmol γ-EC per g fresh weight; Figure 3a–c,e).

Physical mapping and characterization of the *nrc1* and *nrc2* mutants

Our HPLC-MS findings suggest that *nrc1* inefficiently converts cysteine into γ-EC, the precursor of GSH and PCs. Initial rough mapping using an F_2 population of a *nrc1* × Landsberg *erecta* (L*er)* backcross located the mutation on chromosome 4, between the nga1107 and ciw7 markers (Figure 4a). Based on the thiol profile of *nrc1*, candidate genes involved in sulfur assimilation and GSH synthesis from this mapping region were PCR-amplified and sequenced. The locus At4g23100 contained a single C → T mutation in the fourth exon causing a Pro → Leu (P214L) change in the amino acid sequence of γ-EC synthetase (γ-ECS), a key enzyme in glutathione biosynthesis (Figure 4a).

To further determine whether this mutation in γ-ECS was responsible for the *nrc1* phenotype, *nrc1* was crossed into the previously characterized γ-ECS allele, *cad2-1* (Howden *et al*., 1995) (Figure 4b). F_1 seedlings from the reciprocal crosses between the recessive *nrc1* and *cad2-1* mutants were Cd hypersensitive, as determined by root elongation assays in the presence of cadmium (Figure 4b). In contrast, in the presence of Cd, seedlings from crosses of wild-type Col-0 (WT) and *nrc1* or *cad2-1* were not Cd hypersensitive (Figure 4b). These results show that *nrc1* is allelic to *cad2-1*.

To further compare the *nrc1* allele with the *cad2-1* allele, we compared the γ-ECS activity in protein extracts obtained from the two mutants versus the activity of γ-ECS in WT extracts. Using HPLC-MS to determine the initial rates of activity of γ-ECS, we determined that WT extracts synthesizes γ-EC at a rate of 74 pmoles –SH min^{-1} mg $protein^{-1}$, whereas *nrc1* and

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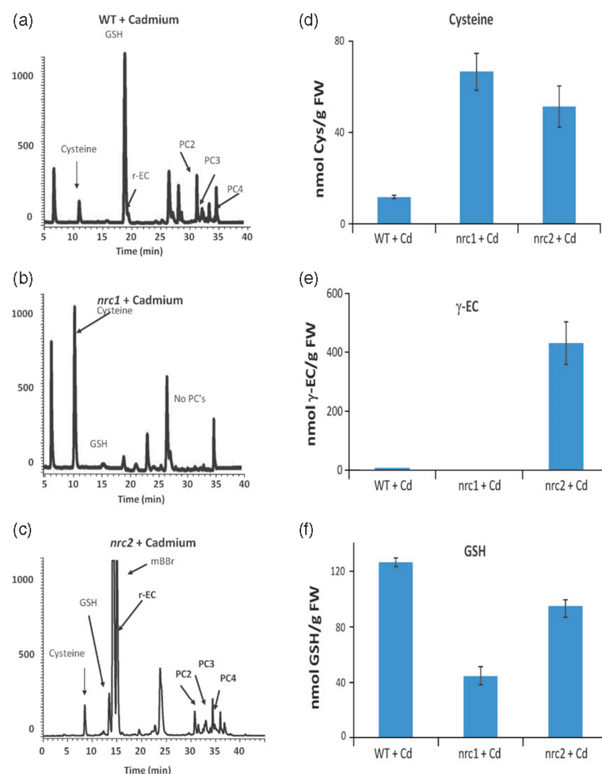

Figure 3. Thiol profile of the *nrc1* and *nrc2* non-response mutants reveals enhanced cysteine levels in *nrc1* and enhanced γ-EC levels in *nrc2*. Fluorescence HPLC-MS was used to identify and quantify thiols
(a) in control plants; (b) in the *nrc1* mutant; (c) in the *nrc2* mutant.
(d) Cysteine quantification using HPLC-MS revealed a five to sixfold accumulation of cysteine in both the *nrc1* and *nrc2* mutants relative to the control following treatment with cadmium.
(e) Fluorescence HPLC-MS also revealed a approximately 50-fold accumulation of γ-glutamylcysteine in *nrc2* relative to the control and a depletion of γ-EC in the *nrc1* mutant (approximately 25% of wild-type levels).
(f) Glutathione quantification using HPLC-MS revealed that both the *nrc1* and *nrc2* mutants are GSH deficient. All bar graphs show the mean of between three and six independent samples. Error bars represent the standard error of the mean (SEM).

cad2-1 synthesize γ-EC at a rate of 13 pmoles –SH min^{-1} mg $protein^{-1}$ (17.56% of the WT rate) and 14 pmoles –SH min^{-1} mg $protein^{-1}$ (18.91% of the WT rate), respectively (Figure 4c). These results suggest that the point mutation in *nrc1* is as severe as the 6-bp deletion found in the *cad2-1* mutant. To further confirm the causative mutation in the *nrc1* mutant, we expressed the genomic *γ-ECS* gene, beginning with the start codon and excluding the 5′ and 3′ untranslated regions (UTRs), ectopically behind the CaMV *35S* promoter in the *nrc1* mutant background. Three independent transformant lines (γ-ECS-Comp1–γ-ECS-Comp3) were selected and T_2 seedlings from these lines were used for root elongation studies and fluorescence HPLC-MS. Ectopic expression of *γ-ECS* in the *nrc1* mutant background rescued the Cd-sensitive root growth phenotype of the *nrc1* mutant (Figure 4d), and greatly decreased the cysteine accumulation phenotype (Figure 4e). Taken together, these results support the conclusion that the identified mutation in *γ-ECS* is the causative mutation in the *nrc1* mutant.

Our HPLC-MS results also suggest that *nrc2* inefficiently converts γ-EC into GSH (Figure 3a,c,f). Genetic mapping using an F_2 population of an *nrc2* × Landsberg *erecta* (L*er*)

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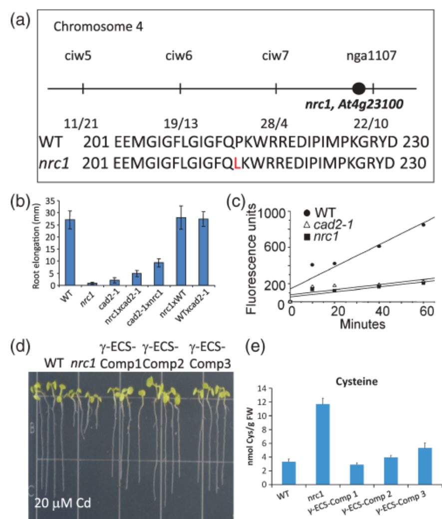

Figure 4. Characterization of the non-response to cadmium mutant *nrc1*.
(a) Mapping of the *nrc1* mutant placed the mutation on chromosome 4 between the ciw7 and nga1107 markers. Candidate gene sequencing identified a point mutation in At4g23100, which causes a Pro → Leu (P214L) change in the γ-glutamylcysteine synthetase protein.
(b) Crosses between *nrc1* and *cad2-1* show the mutants are allelic. The short-root phenotype of the *nrc1* and *cad2-1* mutants grown on 20 μM cadmium (Cd) is not rescued in the *nrc1* × *cad2-1* or the *cad2-1* × *nrc1* F_1 cross, but crossing either mutant into WT restores root elongation in the F_1 generation.
(c) *nrc1* is deficient in γ-EC synthetase (γ-ECS) activity. An *in vitro* assay was performed to determine the activity of the γ-ECS protein from wild-type (WT), *nrc1* and *cad2-1* plants. γ-EC synthetase activity was measured by tracking γ-EC appearance using HPLC-MS in crude extracts (rosette leaves) obtained from wild-type Col-0 (full circles), *nrc1* (full squares) and *cad2-1* (empty triangles). Extracts obtained from WT plants showed a steady synthesis of γ-EC during the assay, whereas *nrc1* and *cad2-1* showed only marginal increases in γ-EC concentration during the assay.
(d) Expression of genomic γ-ECS in the *nrc1* mutant behind a constitutive promoter restores root elongation on Cd. Three independent T_2 transformants (Comp1–Comp3) were used for root elongation experiments on 20 μM Cd.
(e) Seven-day-old complementation lines (γ-ECS Comp1– Comp3) were also used for fluorescence HPLC-MS analyses following 48 h of 100 μM Cd treatment. Cysteine levels in the *nrc1* mutant are three to sixfold higher than in the WT (66.6 ± 8.22 nmol Cys per g fresh weight in *nrc1* compared with 11.8 ± 0.71 nmol Cys per g fresh weight in WT), whereas all three of the γ-ECS complemented (γ-ECS-Comp) lines show a WT cysteine accumulation. All bar graphs show the mean of between three and six independent samples. Error bars represent the standard error of the mean (SEM).

backcross located the mutation on chromosome 5, between the T21B4 and F15F15 markers (Figure 5a). Based on the thiol profile of *nrc2*, candidate genes involved in sulfur assimilation and GSH synthesis from this mapping region were PCR amplified and sequenced. The locus At5g27380 contained a single C → T mutation in the 10th exon, causing an Ala → Val (A404V) change in the amino acid sequence of glutathione synthetase (*GS*), the final enzyme in glutathione biosynthesis (Figure 5a).

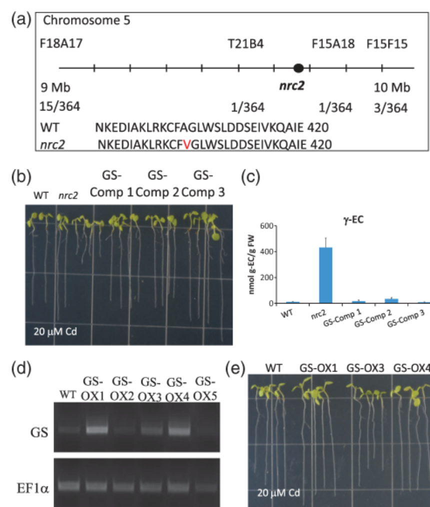

Figure 5. Characterization of the *nrc2* non-response to the cadmium mutant as a first recessive viable allele in the *Arabidopsis* glutathione synthetase gene.
(a) Physical mapping of the *nrc2* mutant placed the mutation on chromosome 5, between the F18A17 and F15F15 markers. Candidate gene sequencing identified a point mutation in At5g23780, which causes an Ala → Val (A404V) change in the glutathione synthetase protein.
(b) Expression of genomic *GS* in the *nrc2* mutant behind a constitutive promoter restores root elongation on Cd. Three independent T_2 transformants (GS Comp1–Comp3) were used for root elongation experiments on 20 μM Cd.
(c) Seven-day-old complementation lines (GS Comp1–Comp3) were also used for fluorescence HPLC-MS analyses following 48 h of 100 μM Cd treatment. Gamma-glutamylcysteine (γ-EC) levels in the *nrc2* mutant are approximately 50-fold higher than in the WT (431.7 ± 72.7 nmol per g fresh weight in *nrc2* compared with 8.57 ± 0.25 nmol per g fresh weight in WT), whereas all three of the *GS* Comp lines show a WT γ-EC accumulation.
(d) The same genomic *GS* construct was also used to transform WT Col-0 plants to produce GS overexpression lines (GS OX1–OX5). RT-PCR of the *GS* gene was performed on T_2 seedlings to identify lines expressing increased *GS* transcript levels compared with WT.
(e) T_2 *GS* overexpression lines (GS-OX1, GS-OX3 and GS-OX4) were then used for root elongation experiments to show that extopic *GS* expression does not increases tolerance to 20 μM Cd. All bar graphs show the mean values from between three and six independent samples. Error bars represent the standard error of the mean (SEM).

No previous viable mutation in the Arabidopsis *GS* gene has been identified, and *GS* T-DNA insertion mutants have been shown to be seedling lethal (Pasternak *et al.*, 2008). Therefore, to determine whether the *GS* point mutation was responsible for the *nrc2* mutant phenotype, the genomic *GS* gene, beginning from the start codon and excluding the

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5′ and 3′ UTR regions, was ectopically expressed behind the CaMV *35S* promoter in the *nrc2* genetic background. Root elongation using T_2 transformant seedlings from three independent transformant lines (GS-Comp1–GS-Comp3) grown on 20 μM Cd confirmed that ectopic expression of the *GS* gene complemented the Cd-sensitive phenotype in the *nrc2* mutant (Figure 5b). Furthermore, 21-day-old soil-grown seedlings appeared less chlorotic than the *nrc2* mutant (Figure S1). Subsequent HPLC-MS analyses of WT, *nrc2* and T_2 seedlings treated with 20 μM Cd show that γ-EC levels were drastically decreased in the complemented lines (Figure 5c). To determine whether this increase in Cd tolerance was an artifact of ectopic expression of GS, we also transformed WT Col-0 with the same GS construct, and selected independent T_2 transformant lines (GS-OX1–GS-OX5). RT-PCR analysis was performed to select lines showing an increase in *GS* transcript (Figure 5d) relative to the WT. We then performed root elongation experiments on 20 μM Cd using these overexpression lines and confirmed that ectopic expression of *GS* does not increase Cd tolerance compared with wild-type lines (Figure 5e). These findings together provide strong evidence that the *nrc2* phenotype is caused by the identified recessive point mutation in *GS*.

Sultr1;2 induction is repressed, even when GSH is depleted

The transcriptional upregulation of sulfate assimilation genes has been described as being part of the plant response to GSH depletion (e.g. PC synthesis during Cd exposure causing GSH depletion; Rouached *et al.*, 2008; Saito, 2004). However, in contrast to this model, the *nrc1* and *nrc2* mutants showed clear GSH depletion (Figure 3d), but failed to produce a strong induction of the SULTR1;2 promoter-driven luciferase reporter (Figure 2d,e) and the native *Sultr1;2* mRNA to wild-type levels after cadmium exposure (Figure 2f,g). These findings point to an alternative hypothesis that the over-accumulation of thiol compounds, either as cysteine (Figure 3d) or γ-EC (Figure 3e), represses the induction of *SULTR1;2* gene expression during Cd exposure in these mutants, even though GSH levels are reduced (Figure 3f). A possible mechanism mediating this response may be that the thiol-dependent cellular redox state also contributes to the Cd-induced gene expression of *Sultr1;2*. To test this hypothesis, we conducted thiol feeding experiments in the presence of cysteine or γ-EC added to the growth medium. Figure 6a shows that the addition of cysteine or γ-EC to the growth media attenuates the induction of *SULTR1;2* gene expression in response to Cd. From the above experiments, however, it was unclear whether the addition of cysteine or γ-EC repressed the luciferase activity by extracellular Cd chelation, metabolite repression or altered cellular redox state. Therefore, we analyzed whether the addition of cysteine, GSH, DTT (a non-physiological thiol) and the non-thiol reducing agent, butylated hydroxyanisole (BHA, which is not known to chelate Cd; Gulcin *et al.*, 2003), altered the Cd-dependent induction of *SULTR1;2* gene expression. As shown in Figure 6b, feeding the non-physiological reducing agents DTT and BHA repressed Cd-induced luciferase activity (Figure 6b) to a similar degree as the metabolites cysteine or GSH (Figure 6b). These results are consistent with a hypothesis where a reducing cellular environment in the *nrc1* and *nrc2* mutants, caused by cysteine or γ-EC over-accumulation, represses Cd-induced gene expression despite low GSH levels in these mutants. Thus, a reducing cellular environment would have hierarchical control, repressing *SULTR1;2* gene induction (Figure 6c). In this experiment, reducing compounds, including the non-physiological reducing agents DTT and BHA, prevent Cd-induced signal transduction, despite the depletion in GSH levels caused by PC production. Furthermore, our results indicate that an increased level of reducing thiols, including cysteine or γ-EC, inhibits Cd-induced *Sultr1;2* gene expression *in vivo*, even when GSH levels are depleted (Figure 6c).

DISCUSSION

A luciferase-based genetic screen was devised using Cd-dependent microarray analysis and the Cd-inducible *Sultr1;2* promoter. In Arabidopsis the *Sultr1;2* transcript is induced by Cd (Rouached *et al.*, 2008) (Figure 1). Microarray analysis (Table S1), RT-PCR analysis (Figures 1a and 2a) and luciferase imaging (Figure 1b,c) demonstrate that the 2.2-kb *SULTR1;2* promoter fragment is a rapid and robust reporter of Cd exposure. Furthermore, we show that the *SULTR1;2* promoter fragment is induced by a well-defined set of metals and metalloids (arsenic), but is induced less well by ROS-inducing agents, such as paraquat (Figure 1c–i).

Our Cd-inducible reporter screen allowed us to identify mutants with decreased, constitutive and increased activity of the reporter gene (Figure 2). These classes of mutants suggest that sulfate uptake in Arabidopsis is regulated by antagonistic transcriptional activators and repressors. To date, one transcriptional regulator of *SULTR1;2*, SLIM1, has been reported and is regulated under sulfur limiting conditions (Maruyama-Nakashita *et al.*, 2006). It remains unknown whether SLIM1 functions in the Cd response, and none of our strong *nrc* mutants mapped to the *SLIM1* gene. The components of the Cd-dependent transcriptional signaling pathway in Arabidopsis remain unknown. Isolation and characterization of the Arabidopsis *nrc* mutants was pursued to advance our understanding of the levels of genetic and mechanistic regulation of the sulfate assimilation pathway that occurs during Cd exposure.

Current model of sulfur homeostasis and GSH depletion during Cd stress

Glutathione is known to be important for mitigating stress as the GSH-deficient mutants *cad2-1*, *pad2*, *rax1-1* and *zir1* have all been identified by their sensitivity to abiotic or biotic

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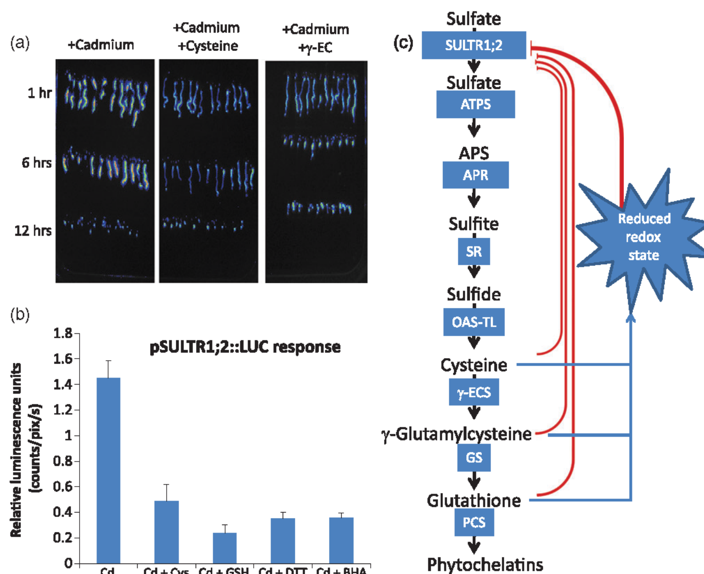

Figure 6. Reducing compounds affect cadmium-dependent induction of the *pSULTR1;2::LUC* reporter.
(a) Addition of 1 mM cysteine or γ-EC to the growth media lowers the response of the control *pSULTR1;2::LUC* line to cadmium over a 12-h time course.
(b) Cadmium-dependent induction of the luciferase reporter gene is affected by reducing compounds. Exposure to 100 μM $CdCl_2$ induces the transcription of the *pSULTR1;2::LUC* reporter gene (Cd). The addition of the reducing compounds cysteine (Cd + 1 mM Cys) or glutathione (Cd + 1 mM GSH) to the growth media strongly represses the cadmium-induced luciferase activity. The non-physiological reducing agents DTT (Cd + 1 mM DTT) and BHA (Cd + 200 μM BHA) also repressed the induction of the reporter gene, suggesting that changes in the cellular redox state is a factor controlling the expression of the *SULTR1;2* gene. All images were quantified after 6 h of exposure to the specified compound.
(c) Schematic representation of putative signals involved in the regulation of the high-affinity sulfate transporter, *SULTR1;2*, during cadmium stress. Glutathione and cysteine are known to repress *SULTR1;2* under many conditions, including sulfur starvation and cadmium stress. Characterization of the *nrc1* and *nrc2* mutants suggests, however, that both GSH demand and changes in cellular redox are required for Cd-dependent transcriptional changes.

stresses (Cobbett *et al.*, 1998; Ball *et al.*, 2004; Parisy *et al.*, 2007; Shanmugam *et al.*, 2011). Another GSH-deficient mutant, *rml*, was identified as lacking a root meristem and having a severe growth and developmental phenotype (Vernoux *et al.*, 2000). These mutants display GSH depletion of various degrees, with the *rml* mutant having the least GSH (approximately 3% of wild-type levels) and *rax1-1* having the highest GSH levels (approximately 50% of wild-type levels) (Vernoux *et al.*, 2000; Ball *et al.*, 2004; Shanmugam *et al.*, 2011). Whereas the severity of these mutations is typically linked to the degree of GSH depletion, Shanmugam *et al.* (2011) have recently shown by systematically analyzing the iron-induced zinc tolerance of each of these mutants that a threshold level of GSH is required for some phenotypes. These results suggest that the phenotypes observed in these mutants may not be linearly correlated with GSH levels alone. Furthermore, the *nrc1* × *cad2-1* F_1 plants (Figure 4b) showed slightly longer root growth than either the *nrc1* mutant or the *cad2-1* mutant alone. This suggests that when the two mutant alleles are expressed together, despite having similar GSH content, the *nrc1* × *cad2-1* F_1 crosses are slightly less sensitive to Cd. One explanation for this observation is that in the *nrc1* × *cad2-1* cross, the γ-ECS dimer is more functional than in the *nrc1* or *cad2-1* offspring (Hothorn *et al.*, 2006; Gromes *et al.*, 2008). This would be consistent with recent findings showing that the regulation of *γ-ECS* in plants is complex and occurs at both the transcriptional and post-transcriptional levels (Hothorn *et al.*, 2006; Gromes *et al.*, 2008).

The current model for sulfur homeostasis in plants proposes that GSH, the most abundant organic thiol in plants, is a strong negative regulator of both sulfate assimilation and cysteine biosynthesis. Glutathione is known to repress the expression and activity of high-affinity sulfate transporters, ATP sulfurylase and APS reductase (Kopriva, 2006). Cellular GSH levels decrease during Cd stress as a result of PC production (Rauser, 1995). This GSH depletion causes an increase in cellular GSH demand, increasing the

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transcription of sulfate uptake-related genes (i.e. *SULTR1;2*) (Kopriva and Rennenberg, 2004; Kopriva, 2006) (Figure 1). This model argues that as sulfate assimilation restores thiol levels, GSH represses sulfate uptake and assimilation genes (Kopriva and Rennenberg, 2004; Kopriva, 2006). Tight regulation of GSH synthesis is needed because of the high reactivity yet essential nature of GSH. Feedback regulation allows the rapid activation of sulfate assimilation during a sudden decrease in GSH levels. This model accounts for many observations, but it assumes that GSH is the major regulator of sulfate assimilation during Cd stress. Growing evidence indicates that cysteine and H_2S are also potent repressors of sulfate transporters; however, it is unclear if cysteine and H_2S repression are direct or mediated through an increase in GSH (Lappartient and Touraine, 1996; Vauclare *et al.*, 2002; Maruyama-Nakashita *et al.*, 2004). Here, by isolating and characterizing mutations that insulate variations in cysteine and γ-glutamylcysteine from changes in GSH concentration, we show the key role of sulfur-containing compounds synthesized prior to GSH production in repressing Cd-induced gene expression (Figure 5c).

nrc mutants reveal cysteine and γ-EC as potent SULTR1;2 repressors

The identification and characterization of the *nrc1* and *nrc2* mutants suggest that another level of Cd-induced gene expression regulation exists. The *nrc1* mutant is GSH deficient but accumulates high levels of cysteine (Figure 3b,d,e), whereas the *nrc2* mutant has decreased GSH levels but accumulates high levels of γ-EC (Figure 4c–e). According to the current model, the GSH status of these mutants should induce *SULTR1;2*, particularly after Cd exposure. Thus, we would expect these mutants to be constitutive or super-response mutants (Figure 2). However, in the *nrc1* mutant, Cd-induced *SULTR1;2* gene expression was strongly repressed, and in the *nrc2* mutant, Cd-induced *SULTR1;2* gene expression was decreased (Figure 2d,e).

We hypothesized that the aberrant accumulation of thiol compounds in the *nrc1* (cysteine) and *nrc2* (γ-EC) mutants also caused a reducing redox environment during Cd exposure, leading to a downregulation of *SULTR1;2* (Figure 6c). Our findings indicate that this reducing cellular state may contribute as a repression mechanism of sulfate assimilation genes in response to Cd stress. Evidence to support this was obtained by feeding experiments with physiological (cysteine and GSH) and non-physiological (DTT) reducing agents, as well as reducing agents not known to chelate Cd (BHA) (Figure 6b). Interestingly, feeding cysteine, γ-EC or GSH to the pSULTR::LUC parental line lowered the Cd-induced luciferase response (Figure 6a,b). Furthermore, feeding non-physiological reducing agents such as DTT and BHA also decreased the *Sultr1;2* induction during Cd exposure (Figure 6b). These results, together with our thiol profiling of isolated genetic mutants, are inconsistent with a solely GSH depletion-driven model, and point to a model where GSH depletion, upstream thiol concentrations and an oxidized cellular redox state are required to induce sulfate assimilation in Arabidopsis in response to Cd stress (Figure 6c). The elevated cysteine or γ-EC thiol levels and reducing cellular conditions in the *nrc1* and *nrc2* mutants are proposed to repress the induction of sulfate assimilation genes, despite the low GSH concentrations in the mutants (Figure 3d). Thus, our results suggest that sulfate assimilation in Arabidopsis is controlled in a hierarchical manner by upstream thiols, the redox state of the cell and the concentration of GSH (Figure 6c). However, oxidative stress alone is not sufficient for mediating Cd-induced *SULTR1;2* expression. Indeed, oxidizing agents such as paraquat (Figure 1f) and H_2O_2 did not induce *SULTR1;2* expression as highly or rapidly as Cd (Figure 1), presumably because they do not cause a decrease in organic thiols despite altering the cellular redox state (Figure 6c).

Regulation of sulfate assimilation and glutathione biosynthesis

The *Sultr1;2* transcript is induced by several stresses, including Cd, attack by pathogens and sulfur deprivation (Maruyama-Nakashita *et al.*, 2006). The *SULTR1;2* promoter was previously used as a reporter gene to screen for mutants unable to induce genes regulated during sulfur starvation (Maruyama-Nakashita *et al.*, 2005). This screen led to the identification of SLIM1 (EIL3), an ethylene insensitive-like transcription factor that regulates the expression of *SULTR1;2* and of genes that mediate glucosinolate synthesis (Maruyama-Nakashita *et al.*, 2006). The SLIM1 protein has been proposed to be a transcriptional activator under conditions of sulfur starvation (Segarra *et al.*, 2009). Presently, there is no direct evidence supporting its role as a transcriptional activator of the *SULTR1;2* promoter. To date it is not known whether SLIM1 directly regulates the expression of *Sultr1;2* or whether its function is independent of *O*-acetylserine (a precursor of cysteine) and GSH concentrations (Maruyama-Nakashita *et al.*, 2006). In summary, the isolation and characterization of the non-response to Cd mutants *nrc1* and *nrc2* points to a new model (Figure 6c) for the regulation of gene expression in response to Cd stress, in which several criteria are necessary for Cd-induced gene expression: (i) GSH depletion; (ii) depletion of upstream thiol levels; (iii) an oxidative cellular redox state, which together control the Cd-induced transcription of *SULTR1;2*.

EXPERIMENTAL PROCEDURES

Arabidopsis accessions

The WT *Arabidopsis thaliana* ecotypes used for mapping were Columbia (Col-0) and Landsberg *erecta* (L*er*-0). The *nrc1* and *nrc2* mutants are in the Col-0 genetic background, and the transformant line pSULTR1;2::LUC is also in the Col-0 genetic background.

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Plant growth conditions

Seeds were sterilized and plated on plates containing quarter-strength MS standard medium (M5519; Sigma-Aldrich, http://www.sigmaaldrich.com), 1 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES), 1% phytoagar (Duchefa, http://www.duchefa.com) and the pH adjusted to 5.6 with 1.0 M KOH (Mäser *et al.*, 2002; Lee *et al.*, 2003). Sterilized nylon mesh with a 200-μm pore size (Spectrum Labs, http://www.spectrumlabs.com) was placed on the surface of the media prior to sowing the seeds. The seeds were then stratified with cold treatment at 4°C for 48 h, and grown under growth room conditions for 5 days (300 μmol m^{-2} s^{-1}, 70% Hr, 16-h light at 21°C/8-h dark at 18°C) (Sung *et al.*, 2007). Seedlings were then transferred to quarter-strength MS, 1 mM MES and 1% agar plates containing 20 μM $CdCl_2$.

Construction of cadmium-response luciferase reporter line

Nine Arabidopsis promoters (2.2 kb before the ATG) from genes found to be induced by cadmium were cloned into the pPZPXomegaL+ vector (kindly provided by Dr. Steve Kay) with *Bam*H1 and/or *Hind*III restriction digestion. The correct orientation of the promoters was confirmed by sequencing the cloned promoter regions in the vector. These nine luciferase constructs were transformed into Arabidopsis and the resulting T_3 homozygous transgenic luciferase plant lines were tested for induction of luciferase protein by monitoring bioluminescence for up to 12 h after exposure to Cd. Of the nine transgenic luciferase reporter lines, several reporter lines containing the promoter of a high-affinity sulfate transporter gene (*SULTR1;2*) showed the most reproducible induction of luciferase, and were hence selected as the reporter lines for the cadmium transcriptional response screening.

Mutant isolation by luciferase luminescence imaging

Homozygous T_3 seeds of a *pSULTR1;2* reporter line were mutagenized with 0.25% EMS for 14 h. The survival of 50% of the mutagenized seeds was confirmed as an indicator of adequate mutagenesis. M_1 seeds were bulk harvested from approximately 6000 M_0 plants and approximately 200 000 M_1 seeds were screened for altered luminescence patterns in response to cadmium treatment (200 μM for 6 h). This is between two and four times more than the minimum M_1 population required to find a mutation in any given G:C pair (Jander *et al.*, 2003). For luciferase imaging, the protocol described by Chinnusamy *et al.* (2002) was followed with the following modifications (Chinnusamy *et al.*, 2002). Seedlings were grown for 5 days horizontally on 36-μm Nitex mesh (Small Parts, Seattle, WA, USA) before being presprayed with 5 mM luciferin (Promega, http://www.promega.com) 6 h before being transferred to either control or treatment plates in order to minimize non-specific luminescence. After transfer the seedlings were subjected to another spraying of 5 mM luciferin and incubated for a period of time (as indicated in the results section) before being imaged using a BERTHOLD NightOWL LB981 imaging system (EG&G Berthold, http://www.berthold.com). A 2-min exposure time was used for capturing the bioluminescent images. Luminescence was quantified using NIGHTOWL.

Total RNA isolation and RT-PCR analysis

Plant materials were flash frozen into liquid nitrogen immediately after treatments. Plant materials were ground using a pre-chilled mortar and pestle. A 100-mg portion of ground plant powder was used to extract total RNA by using a commercial RNA extraction kit (Qiagen, http://www.qiagen.com) (Sunarpi *et al.*, 2005). The quantity and quality of total RNA was recorded using spectrophotometry and gel electrophoresis. A 5-μg portion of total RNA was treated with DNase1 (Ambion, now Invitrogen, http://www.invitrogen.com) to remove DNA contamination from total RNA samples. Prior to the reverse-transcription reaction, DNase-treated total RNA was heated to 65°C for 10 min and immediately cooled down in ice to minimize the secondary structures of total RNAs. A 1-μg portion of total RNA was reverse transcribed with a *Not*I-$d(T)_{18}$ primer using a First Strand cDNA kit (GE Healthcare, http://www.gehealthcare.com) for 60 min at 37°C. Reverse-transcribed cDNA was subjected to PCR to amplify the expression signal of each gene, with the following typical conditions: initial denaturation of cDNA/RNA and inactivation of reverse transcriptase at 95°C for 5 min, then DNA amplification with 25–40 cycles of 95°C for 15 s, 52°C for 15 s, 72°C for 1 min, then a final extension at 72°C for 5 min using an MJ Research PTC 100 Thermal Cycler (GMI, http://www.gmi-inc.com). As a loading control, elongation factor-1α (EF-1α) mRNA was analyzed. Table S2 contains a complete list of all primers used in this study.

Thiol measurements by fluorescence HPLC

Thiol-containing compounds in plant samples, including cysteine, γ-EC, GSH and PCs, were analyzed using fluorescence detection HPLC, as described by Fahey and Newton (1987). To analyze the levels of thiol compounds produced by plants in response to treatment, plants were grown on minimal growth media plates for 5 days then transferred to fresh media plates containing 200 μM cadmium. In order to minimize the oxidation of thiol compounds during the extraction, plant seedlings were flash-frozen in liquid nitrogen, and then ground and extracted as previously described (Sung *et al.*, 2009). The peaks of thiol compounds were identified by coupled parallel mass spectrometry measurement, as previously described (Chen *et al.*, 2006), and quantified using XCALIBUR (Thermo Scientific, http://www.thermoscientific.com). To identify the peptides from plant extracts, PC2, PC3 and PC4 standards were synthesized on a MILLIGEN 9050 PepSynthesizer (Millipore, http://www.millipore.com) using Fmoc-Glu-OtBu (Bachem, http://www.bachem.com). Other thiol standards, such as glutathione, cysteine, γ-EC and NAC, were purchased from Sigma-Aldrich. All reported thiol quantities are means of between three and nine biologically independent samples, and error bars indicate the standard error of the mean (SEM).

Plant growth conditions and cadmium treatment

For plate-based assays, including heavy metal treatments, luciferase luminescence assay and plant growth for RT-PCR analysis, seeds were germinated on nylon mesh (Spectrum Laboratories Inc., http://www.spectrumlabs.com) on quarter-strength MS media and grown for 1 week at 22°C, 75% humidity, with a 16-h light/8-h dark photoperiod regime at approximately 75 μmol m^{-2} s^{-1} light intensity in a Conviron growth chamber (Controlled Environments Inc., http://www.conviron.com). Seedlings on nylon mesh were transferred either to treatment or control plates and incubated for up to 12 h, as previously described (Sung *et al.*, 2009).

Feeding experiments

We performed feeding experiments using the control pSULTR1;2::LUC reporter line (Figure 1). We exposed 5-day-old seedlings to 100 μM Cd, 100 μM Cd + 2500 μM Cys, 100 μM Cd + 2500 μM DTT, 100 μM Cd + 300 μM BHA or 100 μM Cd + 2500 μM GSH for 6 h (Figure 4e), before performing luciferase imaging on the roots and quantifying the relative luciferase luminescence.

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nrc1 and *cad2-1* enzyme activity experiments

The activity of γ-ECS was measured in protein extracts using HPLC fluorescence and thiol-derivatization with monobromobimane, using a modified version of the protocol described by Hell and Bergmann (1990). Rosette leaves from wild-type (Col-0), *nrc1* or *cad2-1* were ground in liquid nitrogen and 100–300 mg of ground tissue were mixed 1:1 with extraction buffer (100 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.0). Unless otherwise stated, all steps were carried out at 4°C and all buffers were saturated with nitrogen prior to being used to minimize the oxidation of thiols during protein extraction and enzymatic activity measurements. The protein extract was centrifuged for 10 min at 10 000 ***g***, and the supernatant was desalted using Sephadex G-25 previously equilibrated with extraction buffer. The enzyme activity assay (500 μl final volume) contained 100 mM Tris (pH 8.0), 50 mM $MgCl_2$, 20 mM glutamate, 5 mM ATP and an ATP regenerating system that consisted of 5 mM phosphoenolpyruvate, 1 mM DTT and 10 U ml^{-1} of pyruvate kinase. The protein extract was incubated for 10 min at 30°C, and γ-ECS activity was started by adding 1 mM cysteine. At defined time points, 50 μl of the reaction solution were taken and thiols were derivatized by adding 40 μl of monobromobimane (2 mM) and incubated for 20 min at 40°C. Derivatization was stopped by adding 10 μl of PCA 30% v/v. Samples were mixed using a vortex and protein was precipitated by centrifugation (10 min at 10 000 ***g***). The supernatant was filtered using 0.45-μm Ultrafree-MC filters (Amicon; Millipore) before being analyzed by HPLC as described previously (Mendoza-Cozatl *et al.*, 2008). The quantity of γ-EC synthesized over time was normalized to the protein content in the plant extract, quantified using the Bradford reagent (Sigma-Aldrich) and BSA as a protein standard.

nrc1 and *nrc2* complementation

All primers used for PCR amplification for cloning are listed in Table S2. For constitutive *γ-ECS* expression in the *nrc1* mutant, a *γ-ECS* genomic DNA fragment was amplified from Col-0 gDNA using the primers TJ199-F and TJ199-R (0–3294 bp). The amplified genomic *γ-ECS* DNA fragment, which excluded both the 5′ and 3′ untranslated regions (UTRs), was cloned into pENTR/D-TOPO® (Invitrogen), following the manufacturer's instructions. The *CaMV 35S*:γ-ECS-NOS construct was obtained by recombining the *γ-ECS* genomic sequence into a Gateway®-compatible pGreenII plasmid (Hellens *et al.*, 2000) containing the $35S_{pro}$ and the *NOS* terminator ($35S_{pro}$:GW-NOS_{ter}), using LR Clonase II® (Invitrogen). The pGREENII *35S:γ-ECS-NOS* construct was transformed using electroporation into *Agrobacterium tumefacians* strain GV3101.

For constitutive *GS* expression in the *nrc2* mutant, a *GS* genomic DNA fragment was amplified from Col-0 gDNA using the primers TJ198-R and TJ198-R (0–2702 bp). The amplified genomic *GS* DNA fragment, which excluded both the 5′ and 3′ UTRs, was cloned into pENTR/D-TOPO® (Invitrogen), following the manufacturer's instructions. The *CaMV 35S*:GS-NOS construct was obtained by recombining the *GS* genomic sequence into a Gateway®-compatible pGreenII plasmid (Hellens *et al.*, 2000) containing the $35S_{pro}$ and the *NOS* terminator ($35S_{pro}$:GW-NOS_{ter}), using LR Clonase II® (Invitrogen). Note that our attempts to complement *nrc2* using a *35S*-driven *GS* cDNA were not successful, whereas the genomic DNA complimented the *nrc2* growth and yellowing phenotype (Figure S1) in six independent lines (all isolated transformants). We tested the complementation of the Cd-dependent root growth and thiol accumulation in three of these lines, as shown in the results section. The pGREENII *35S:GS-NOS* construct was transformed using electroporation into *Agrobacterium tumefacians* strain GV3101.

Arabidopsis thaliana was transformed using the floral-dip method (Clough and Bent, 1998) with the GV3101 strains described above. The helper plasmid, pSoup, was used for the pGreenII-carrying strains (Hellens *et al.*, 2000). The pGREENII *35S:GS-NOS* construct was used to transform the *nrc2* mutant for complementation and Col-0, whereas the pGREENII *35S:γ-ECS-NOS* construct was used to transform the *nrc1* mutant for complementation. Hygromycin selection of transformants was performed in both the T_1 and T_2 generations.

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SUPPORTING INFORMATION

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

Figure S1. Visual phenotype of 20-day-old *nrc2* mutant plants grown in soil.

Table S1. A list of the 30 genes most influenced by cadmium exposure, ranked in order of decreasing induction.

Table S2. A list of primers used for RT-PCR, promoter cloning, gene sequencing and complementation experiments.

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Appendix 2:

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

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

David G Mendoza-Cózatl[1], Timothy O Jobe, Felix Hauser and Julian I Schroeder

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* metals, at high concentrations, 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 posses 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.

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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 [3–5,6•]. 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 (Figure 1) [1–5,7•].

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. Figure 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 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, Figure 1) [8••,9••]. *S. pombe* Abc2 is able to suppress the cadmium hyper-sensitivity and restore 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 wild-type plants [9••]. Interestingly, ABCC1 and ABCC2 were previously also described as 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 (Figure 2, Supplementary Table 1). It should be noted, however, that *S. pombe* Abc2 and *Arabidopsis* ABCC2 share ≈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-GS_2 is one of Hmt1 substrates or 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 [27–29,30•,31••,32]. Uptake of metals and arsenic into roots and the xylem has 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 (Figure 1). Arsenate [As(V)] is taken up and loaded into the xylem by phosphate transporters [33] while arsenite [As(III)] uptake and translocation are 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 the 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 [38–40], 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 co-ordinated with thiol-containing ligands (Figure 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(loids) through the phloem. The plasma membrane transporters that load PC-metal(loid) complexes into the phloem remain unknown (Figure 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. Figure 2A). Companion cells and sieve elements (phloem) are connected through highly permeable plasmodesmata

Figure 1

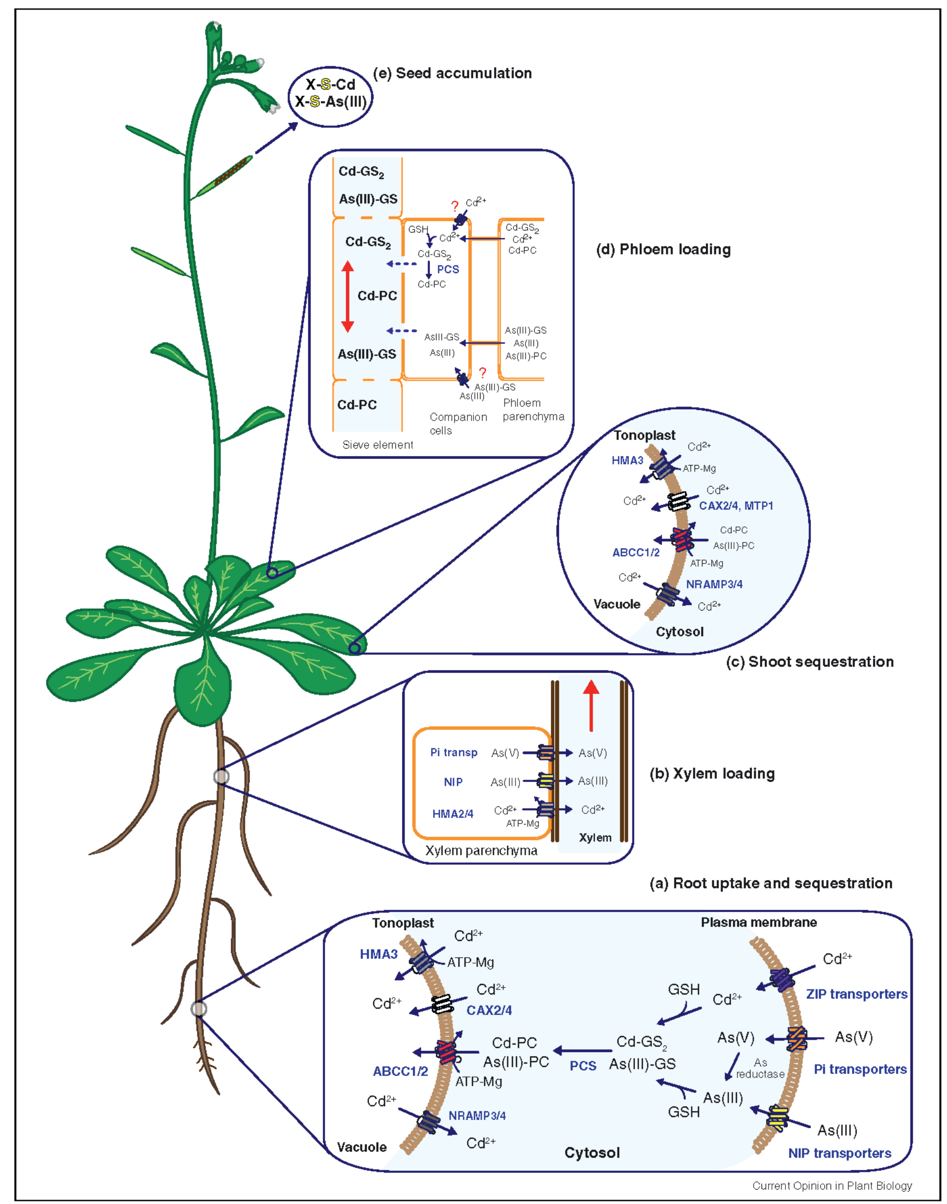
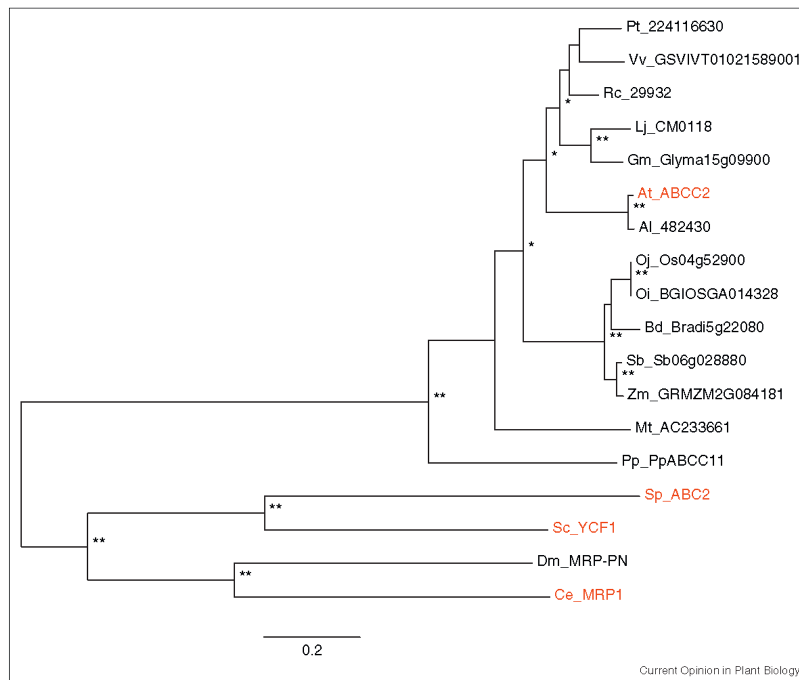


Figure 2



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 phytochelatins: *P. patens*, *S. cerevisiae* and *D. melanogaster*. The tree was constructed using phyml 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 90–100%. The scale bar indicates 0.2 substitutions per site.

(Figure 1 Legend) 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). Because of the high permeability of companion cells and sieve element plasmodesmata, GSH, As(III)-induced 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].

[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, Figure 1) [35,45–47]. We have analyzed low molecular weight thiols in *Arabidopsis* seeds and found significant levels of GSH but not PCs (Mendoza-Cozatl, 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. Figure 2B).

But why are PCs 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 by reducing toxic metal(loid) accumulation.

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. PCs mediate the detoxification of Cd, As, and Hg, and plants deficient in PC synthesis are highly sensitive to these metal(loids). 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: first, enhanced metal(loid) uptake in roots, second, reduced sequestration of metals in root vacuoles, third, enhanced root-to-shoot translocation and fourth, enhanced sequestration of metals in shoot vacuoles (Figure 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 suboptimal 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 μM) and high (50 μM) 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 (Figure 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 TFs 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 function NRT1.8 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 TFs mediate early Cd and As transcriptional regulation are open questions.

Concluding remarks

Metal accumulation and homeostasis require the co-ordination of several processes working simultaneously to regulate uptake, long-distance transport and distribution of metals to different cells and tissues (Figure 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 entry 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbi.2011.07.004.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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6. • Duffus JH: **Heavy metals' a meaningless term?** *Pure Appl Chem* 2002, **74**:793-807.
Metals with a density higher than five (5 g/mL) have been historically called 'heavy metals'. There is some discussion about whether this term should be replaced by other terms such as trace metals or trace metallic elements. This IUPAC report explains some of the reasons why the term 'heavy metal' may be misleading or even inaccurate. For simplicity and to avoid further confusion, our review refers only to metals and metalloids and emphasizes which of them are essential for biological functions and which ones are not.

7. • Buescher E, Achberger T, Amusan I, Giannini A, Ochsenfeld C, Rus A, Lahner B, Hoekenga O, Yakubova E, Harper JF *et al.*: **Natural genetic variation in selected populations of *Arabidopsis thaliana* is associated with ionomic differences**. *PLoS ONE* 2010, **5**:e11081.
The authors use inductively coupled plasma — mass spectroscopy to establish the 'ionome' of 12 different Arabidopsis accessions and three recombinant inbred lines. They performed elemental analysis on 17 different elements and compared the results to identify QTLs linked to differences in the ionomic profiles of these accessions. The ionomic data have been made publically available and is an excellent resource for comparing the impact of both genetics and various environmental conditions on the elemental composition of Arabidopsis.

8. •• Mendoza-Cozatl DG, Zhai Z, Jobe TO, Akmakjian GZ, Song WY, Limbo O, Russell MR, Kozlovskyy VI, Martinoia E, Vatamaniuk OK *et al.*: **Tonoplast-localized Abc2 transporter mediates phytochelatin accumulation in vacuoles and confers cadmium tolerance**. *J Biol Chem* 2010, **285**:40416-40426.
The authors identify a novel vacuolar phytochelatin–cadmium uptake transporter in a subfamily different from the previously identified phytochelatin transporter Hmt1. In contrast to the hmt1 mutant, which still accumulates a significant amount of phytochelatins in vacuoles, the hmt1abc2 double mutant largely removes phytochelatin accumulation in vacuoles. A new function for HMT1 in Cd–S cluster transport is also proposed.

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Plant vacuolar phytochelatin transporters were sought for more than 15 years. In this paper the authors identified two Arabidopsis vacuolar ABC transporters required for arsenic tolerance. *In vitro* experiments show that ABCC1 and ABCC2 mediate the uptake of PC and PC-As(III) complexes into plant vacuoles.

10. •• Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF: **Gene limiting cadmium accumulation in rice**. *Proc Natl Acad Sci U S A* 2010, **107**:16500-16505.
By using QTL mapping, the authors identify a gene responsible for reducing cadmium accumulation in grains of the Nipponbare rice cultivar. OsHMA3 is expressed at the root tonoplast and the cultivar showing enhanced expression of functional HMA3 in roots significantly reduces accumulation of cadmium in seeds and shoots.

11. • Li J-Y, Fu Y-L, Pike SM, Bao J, Tian W, Zhang Y, Chen C-Z, Zhang Y, Li H-M, Huang J *et al.*: **The arabidopsis nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance**. *Plant Cell* 2010, **22**:1633-1646.
The authors report the functional characterization of NRT1.8 as a sought after gene mediating nitrate unloading from the xylem. The mutant was also found to be Cd hypersensitive suggesting that nitrate levels in the xylem and distribution in plants plays a role in cadmium tolerance.

12. • Ueno D, Milner MJ, Yamaji N, Yokosho K, Koyama E, Clemencia Zambrano M, Kaskie M, Ebbs S, Kochian LV, Ma JF: **Elevated expression of TcHMA3 plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens***. *Plant J* 2011, **66**:852-862.
Thlaspi caerulescens is considered a Cd/Zn hyperaccumulator. The authors show that the enhanced cadmium accumulation of a hyperaccumulating ecotype of *T. caerulescens* correlates well with an enhanced gene copy and expression of TaHMA3. TaHM3 is functional in both ecotypes, but in the ecotype showing higher tolerance and cadmium accumulation, TaHMA3 exhibits constitutive expression at higher levels.

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