

# Vacuolar Transporters for Cadmium and Arsenic in Plants and their Applications in Phytoremediation and Crop Development

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**Soil contamination by heavy metals and metalloids such as cadmium (Cd) and arsenic (As) poses a major threat to the environment and to human health. Vacuolar sequestration is one of the main mechanisms by which plants control toxic materials including Cd and As. Understanding the mechanisms of heavy metal tolerance and accumulation can be useful for both phytoremediation and safe crop development. In this review, we summarize recent advances in deciphering the molecular mechanisms underlying vacuolar sequestration of Cd and As, and discuss potential biotechnological applications of this knowledge and efforts towards attaining these goals.**

**Keywords:** Arsenic • Cadmium • Phytoremediation • Safe crop • Vacuolar transporter.

**Abbreviations:** ABCC, ATP-binding cassette subfamily C protein; ACR3, Arsenic Compounds Resistance 3; ATQ1, arsenate tolerance QTL1; CaMV, *Cauliflower mosaic virus*; CAX, Ca<sup>2+</sup> exchanger; GPL4, GLABRA1 ENHANCER BINDING PROTEIN transcription factor; GSH, glutathione; GWAS, genome-wide association study; HAC1, High Arsenic Content 1; HIR1, heavy metal induced RING E3-ligase; HMA, heavy metal ATPase; MAN3, endo- $\beta$ -mannanase 3; NRAMP, natural resistance-associated macrophage protein; PC, phytochelatin; PCS, phytochelatin synthase; QTL, quantitative trait locus; ROS, reactive oxygen species; SnRK2, SNF1-related protein kinase 2; YCF1, yeast cadmium factor 1; ZIP, zinc-regulated transporters, iron-regulated transporter-like protein.

## Introduction

As the human population increases and industrialization booms to support the growing population, environmental pollution has become an urgent problem for human beings. Many cases have been reported where toxic pollutants in the environment have been shown to have a serious negative impact on various living organisms (Jaishankar et al. 2014, Bartrons et al. 2016). This is particularly true for humans, who are at the top of the food chain. Most organic pollutants are degraded over time,

mostly by bacteria, but inorganic pollutants are not degraded, and accumulate in the environment. Inorganic pollutants include heavy metals [e.g. cadmium (Cd), lead (Pb), chromium (Cr), mercury (Hg) and nickel (Ni)] or metalloids [e.g. arsenic (As)] that cause environmental pollution and severely inhibit plant growth and development, and thus decrease the productivity of crop plants (Clemens 2006). These metal and metalloid contaminants originate from either natural or anthropogenic sources, such as industrial, agricultural and mining activities (Fasani et al. 2018).

Cd and As have since long ranked high on the US Agency for Toxic Substances and Disease Registry (ATSDR) Priority List of Hazardous Substances (<https://www.atsdr.cdc.gov/spl/index.html>). During the past decade, tremendous advances have been made in our understanding on the mechanisms of metal accumulation and tolerance in plants (reviewed in Verbruggen et al. 2009, Kramer 2010, van der Ent et al. 2013, Clemens and Ma 2016). Here, we focus on recent advances in the area of vacuolar transporters that are important for the accumulation of and resistance to Cd and As in plants. In addition, we discuss how knowledge gleaned on this topic can be used to develop crops that are free from contamination, and for phytoremediation, a technology that uses plants to clean up the polluted environment.

## Vacuolar Transporters for Cd and As

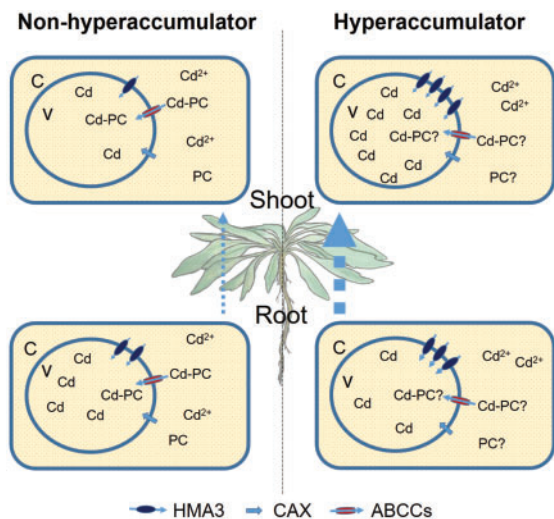
The vacuole is the largest organelle inside the mature plant cell. It is an important reservoir of ions and metabolites, and is crucial for the detoxification process as well as normal cell development (Marty 1999, Martinoia et al. 2012). Toxic substances such as Cd and As are mainly stored in the vacuole, as a means of detoxifying the cytosol (Table 1).

## Cadmium transporters (Fig. 1)

Several families of transporters, such as heavy metal ATPases (HMAs), Ca<sup>2+</sup> exchangers (CAXs), natural resistance-associated macrophage proteins (NRAMPs) and ATP-binding cassette subfamily C proteins (ABCCs), have been reported to be involved in the vacuolar sequestration of Cd (Korenkov et al.

**Table 1** Transporters involved in As and Cd transportation across the tonoplast

Transporter family	Transporters	Plant species	Possible substrates	References
ABCC	AtABCC1, AtABCC2	<i>A. thaliana</i>	PC-As, apo-PC, PC-Cd	Song et al. (2010); Park et al. 2012
	AtABCC3	<i>A. thaliana</i>	PC-Cd	Brunetti et al. (2015)
	OsABCC1	<i>O. sativa</i>	PC-As, apo-PC	Song et al. (2014)
HMA	AtHMA3	<i>A. thaliana</i>	Cd, Zn, Pb, Co	Gravot et al. (2004); Morel et al. (2009); Chao et al. (2012)
	OsHMA3	<i>O. sativa</i>	Cd	Miyadate et al. (2011); Ueno et al. (2010)
	GmHMA3	<i>G. max</i>	Cd	Wang et al. (2012)
	NcHMA3	<i>N. caerulescens</i>	Cd	Ueno et al. (2011)
	SaHMA3	<i>S. alfredii</i>	Cd	J. Zhang et al. (2016)
	SpHMA3	<i>S. plumbizincicola</i>	Cd	Liu et al. (2017)
CAX	AtCAX2, AtCAX4	<i>A. thaliana</i>	Ca, Cd, Zn, Mn	Korenkov et al. (2007, 2009)
	AhCAX1	<i>A. halleri</i>	Cd	Baliardini et al. (2015)
	SaCAX2	<i>S. alfredii</i>	Cd	M. Zhang et al. (2016)
NRAMP	AtNRAMP3, AtNRAMP4	<i>A. thaliana</i>	Cd, Fe, Mn	Lanquar et al. (2005)
	NcNRAMP3, NcNRAMP4	<i>N. caerulescens</i>	Cd, Fe, Mn	Oomen et al. (2009)
ACR3	PvACR3	<i>P. vittata</i>	As(III)	Indriolo et al. (2010)



**Fig. 1** Vacuolar transporters of Cd. In non-hyperaccumulator plants, such as *Arabidopsis* and rice, Cd is sequestered into the vacuoles of the root by HMA3, CAX and ABCC transporters. The root sequestration of Cd limits the amount of root to shoot translocation of Cd (thin blue dotted arrow). However, in Cd hyperaccumulators, such as *N. caerulescens*, *A. halleri*, *S. alfredii* and *S. plumbizincicola*, the majority of Cd is transported to the shoot (thick blue dotted arrow), and sequestered in the vacuole of leaf cells. The elevated expression of HMA3 plays a major role in Cd sequestration in these plants. V, vacuole; C, cytosol.

2007, Park et al. 2012, J. Zhang et al. 2016). HMA3, a P-type heavy metal ATPase, mediates the vacuolar sequestration of various heavy metals, including zinc (Zn), Cd, Pb and cobalt (Co), in different plant species (Gravot et al. 2004, Morel et al. 2009, Ueno et al. 2010, Ueno et al. 2011, J. Zhang et al. 2016, Liu et al. 2017). However, the detailed functions of HMA3 vary in different plants, in terms of substrate specificity, expression pattern and expression level. *Arabidopsis* HMA3 has a broad substrate specificity, transporting Zn, Cd, Pb and Co into the

vacuole (Morel et al. 2009). A genome-wide association study (GWAS) of the natural variation of foliar Cd concentration in different *Arabidopsis* accessions demonstrates that HMA3 is a major determinant of leaf Cd accumulation (Chao et al. 2012). In *Arabidopsis*, HMA3 is mainly expressed in the root, and controls the foliar Cd concentration by sequestering Cd in the root and limiting long-distance transport of Cd from the root to the shoot (Chao et al. 2012). Interestingly, the most commonly used *Arabidopsis* ecotype, Col-0, most probably contains an inactive form of HMA3, since a frameshift has been reported after amino acid 542 (Morel et al. 2009, Chao et al. 2012), resulting in large difference in both Cd tolerance and accumulation compared with most other ecotypes (Chao et al. 2012). Quantitative trait locus (QTL) analysis of low and high Cd-accumulating *Oryza sativa* (rice) cultivars also identified HMA3 as controlling shoot Cd accumulation (Ueno et al. 2010, Miyadate et al. 2011). In a low Cd-accumulating cultivar of rice, OsHMA3 functionally and specifically sequesters Cd into vacuoles in the root. However, in the high Cd-accumulating cultivar, the transporter is not active due to a single amino acid mutation (Ueno et al. 2010, Miyadate et al. 2011). Although overexpression of OsHMA3 in rice results in increased Zn concentrations in the roots, it might have been caused by increased expression of ZIP (zinc-regulated transporters, iron-regulated transporter-like protein) family transporter genes, such as ZIP4, ZIP5, ZIP8, ZIP9 and ZIP10 (Sasaki et al. 2014). A similar result was found in *Glycine max* (soybean) plants (Wang et al. 2012). In contrast to non-hyperaccumulators, such as *Arabidopsis*, rice and soybean, Cd hyperaccumulators, such as *Noccaea caerulescens*, *Arabidopsis halleri*, *Sedum alfredii* and *Sedum plumbizincicola*, are able to accumulate extremely high concentrations of Cd in their shoots without showing any toxicity symptoms. In *N. caerulescens*, high expression of NcHMA3, due mainly to the increased copy number of the gene, plays a major role in Cd hyperaccumulation (Ueno et al. 2011). Similarly, in the hyperaccumulating ecotype *S. alfredii*, SaHMA3h is highly expressed and has an increased copy

number of the gene compared with its non-hyperaccumulator ecotype (J. Zhang et al. 2016). Recently, Liu et al. (2017) found that SpHMA3 is critical for Cd detoxification in *S. plumbizincicola* shoots, and acts by sequestering Cd into vacuoles, particularly in young leaf cells. Slightly different from *N. caerulescens*, which has similar expression levels of HMA3 in the root and shoot (Ueno et al. 2011), *SaHMA3h* and *SpHMA3* exhibit much higher expression levels in the shoot than in the root (J. Zhang et al. 2016, Liu et al. 2017). The high expression of *SaHMA3h* and *SpHMA3* in the shoot could increase the accumulation of Cd in the shoot, rendering the shoot a Cd reservoir, whereas the relatively low expression of these genes in the root could maintain the high root to shoot translocation efficiency. HMA3s from both *N. caerulescens* and *S. alfredii* exhibit high substrate specificity for Cd, over Zn, Pb or Co (Ueno et al. 2010, J. Zhang et al. 2016). However, the mechanisms that determine the substrate specificity of HMA3 have yet to be identified.

The CAXs are tonoplast-localized transporters that are mostly calcium (Ca) specific. However, AtCAX2 and AtCAX4 have been shown to transport not only Ca but also other metals, including Cd, Zn and manganese (Mn) (Korenkov et al. 2007, Korenkov et al. 2009). In the Cd hyperaccumulator *A. halleri*, AhCAX1 was shown to co-segregate with Cd tolerance (Baliardini et al. 2015), suggesting that AhCAX1 may contribute to Cd tolerance in this plant. A CAX2-like protein, SaCAX2, present in a hyperaccumulating ecotype of *S. alfredii* confers Cd tolerance and accumulation when heterologously expressed in yeast and tobacco plants (M. Zhang et al. 2016).

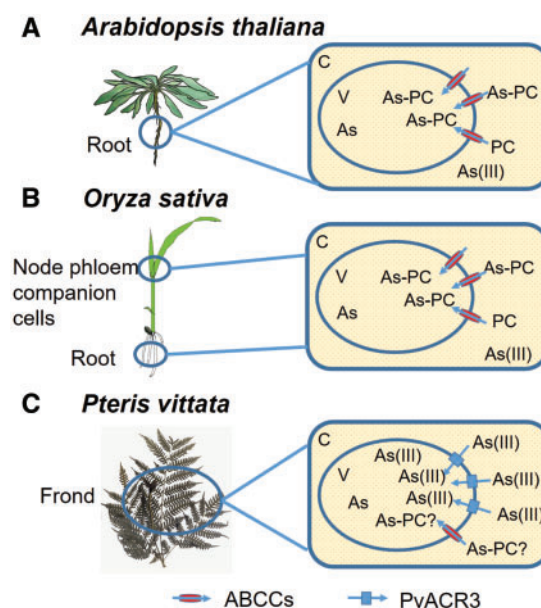
The NRAMPs also transport various divalent transition metals, including iron (Fe), Mn, Zn and Cd. Among the six NRAMPs present in *A. thaliana*, NRAMP3 and NRAMP4 are localized at the tonoplast and export metals from the vacuole to the cytosol, thus playing a role in the remobilization of essential metals from the vacuole to the cytosol (Lanquar et al. 2005). The *atnramp3 atnramp4* double knockout plant exhibits increased sensitivity to Cd (Oomen et al. 2009), accompanied by Fe and Mn deficiency symptoms. The increased Cd sensitivity is probably due to Cd inhibiting the uptake of Fe and Mn, and thus exacerbates the symptoms caused by the defective Fe and Mn homeostasis.

In contrast to HMA3, the CAX and NRAMP transporters, which transport the ionic form of Cd ( $Cd^{2+}$ ), and the ABCC transporters, which belong to the ATP-binding cassette transporter superfamily, transport Cd conjugated to phytochelatin (PCs). The *Arabidopsis abcc1abcc2* double mutant exhibits a Cd-sensitive phenotype. Staining with a Cd-sensitive dye revealed that Cd is mainly localized in the cytosol of the mesophyll protoplasts of the double mutant, whereas almost all Cd occurs in the vacuoles of wild-type cells (Park et al. 2012). These data suggest that ABCC1 and ABCC2 transporters are important for Cd sequestration into the vacuole (Park et al. 2012). Recently, AtABCC3 was also found to play a role in PC-mediated Cd tolerance (Brunetti et al. 2015). Cd treatment induces the expression of AtABCC3. A mutant defective in AtABCC3 shows increased Cd sensitivity; in contrast, plants overexpressing AtABCC3 exhibit enhanced Cd tolerance.

When AtABCC3 is expressed in the *cad1-3* mutant, which is defective in PC synthesis, or when the AtABCC3-overexpressing plants are subjected to 1-buthionine sulfoximine (BSO; an inhibitor of PC biosynthesis) treatment, there is no increase in Cd tolerance, which suggests that the function of AtABCC3 is dependent on PCs (Brunetti et al. 2015). The reason why HMA3, but not an ABCC transporter, was identified in a GWAS study as the major determinant for leaf cadmium is likely to be due to the largely redundant functions of ABCC1 and ABCC2, and to the low probability that both genes are mutated in the same plant.

### Arsenic transporters (Fig. 2)

Inside the vacuole, As is mainly sequestered in the form of PC–As or GSH (glutathione)–As conjugates. Thus, the transporters responsible for vacuolar sequestration of PC–As or GSH–As conjugates play a critical role in As detoxification. As conjugates transporters in plants were expected to belong to the ABC transporter family, since As transporters were shown to belong to this family of transporters in both humans and budding yeast: in humans, the bisGSH–As(III) complex is transported by two ABC transporters, HsABCC1 and HsABCC2 (Leslie et al. 2004), and in *Saccharomyces cerevisiae* by ScYCF1 (yeast cadmium factor) (Ghosh et al. 1999). In plants, the detoxification of As is mainly dependent on PCs (Zhao et al. 2009);



**Fig. 2** Vacuolar transport mechanisms of As. As sequestration in the vacuole is mainly controlled by ABCC transporters in angiosperms. In *Arabidopsis*, AtABCC1 and AtABCC2 function redundantly as As–PC conjugates transporters, which sequester As–PC into root vacuoles and confer As tolerance. In rice, OsABCC1 plays a major role in the vacuolar sequestration of As. OsABCC1 sequesters As–PC conjugates into the vacuoles of root cells and node phloem companion cells, and thus controls As accumulation in the rice grain as well as As tolerance. However, in *Pteris vittata*, an As hyperaccumulator, As is mainly sequestered in the vacuole in the form of As(III) by PvACR3. V, vacuole; C, cytosol.

thus, the vacuolar transporter for As is likely to be a PC–As transporter rather than a GSH-conjugated As(III) transporter.

AtABCC1 and AtABCC2 were originally reported to sequester GSH-conjugated organic molecules into plant vacuoles (Lu et al. 1997, Lu et al. 1998). The importance of these ABC transporters in As detoxification was later revealed by Song et al. (2010); AtABCC1 and AtABCC2 are major vacuolar PC–As conjugate transporters in Arabidopsis. The *abcc1abcc2* double knockout is hypersensitive to various forms of As, and exhibits a dramatic reduction of vacuolar uptake of PC–As (Song et al. 2010). Transport assays showed that AtABCC1 and AtABCC2 could transport both PC–As conjugates and apo-PC. In addition, the *abcc1abcc2* double mutant also shows hypersensitivity to Cd and Hg (Park et al. 2012). Rice has only one ortholog of AtABCC1 and AtABCC2, OsABCC1, which plays an important role in reducing As accumulation in rice grains (Song et al. 2014). Expression of OsABCC1 complements the As hypersensitivity of the *abcc1abcc2* double mutant and confers PC-dependent As tolerance in yeast. OsABCC1 is ubiquitously expressed in rice tissues, and slightly up-regulated by treatment with high concentrations of As. In the nodes, OsABCC1 localizes at the phloem region of the vascular bundles. An OsABCC1 loss-of-function rice mutant is more sensitive to As and contains higher concentrations of As in the grain, but lower concentrations of As in the nodes, than its wild-type background plant. OsABCC1 localizes at the tonoplast. Together, these data indicate that OsABCC1 limits As translocation to the grain by sequestering As into the vacuoles of the phloem companion cells of the nodes in rice (Song et al. 2014). Direct transport assays were not conducted, and thus it is not known whether OsABCC1 transports apo-PC, As-conjugated PC or both forms.

Ferns have mechanisms for sequestering As into the vacuole that are distinct from those in angiosperms. In the Chinese brake fern (*Pteris vittata*), the first As hyperaccumulator plant identified, As enters root cells via phosphate transporters in the form of arsenate [As(V)]. Once taken up by the root, arsenate is reduced to arsenite [As(III)], which is the primary form of As that is translocated to the fronds, where it is stored in vacuoles. In *P. vittata*, PvACR3 (Arsenic Compounds Resistance 3), a homolog of the yeast As exporter ScACR3, is associated with As tolerance (Indriolo et al. 2010). PvACR3 can rescue the As sensitivity of the *acr3* yeast mutant. The reduced As tolerance of the RNA interference (RNAi) lines of PvACR3, together with its tonoplast localization, suggests that PvACR3 functions in the vacuolar sequestration of As in *P. vittata*. ACR proteins exist in several plant species, but not in angiosperms. Although the amino acid sequence of PvACR3 shows a high similarity to yeast ACR3 (ScACR3), these proteins have different subcellular localizations. ScACR3 is an As exporter localized at the plasma membrane that transports [As(III)] out of the cell (Wysocki et al. 1997). In contrast, PvACR3 localizes at the vacuolar membrane and sequesters [As(III)] into the vacuole in *P. vittata*. Interestingly, when PvACR3 is overexpressed in Arabidopsis, it localizes at the plasma membrane and exports As out of the root, thus enhancing As tolerance (Chen et al. 2013).

## Other Factors that Assist/Regulate the Cd and As Transporters

### Metal(loid) chelators

After entering cells, metals and metalloids form complexes by binding with metal ligands, including GSH, PCs, metallothioneins, nicotianamine, organic acids and amino acids (Callahan et al. 2006). GSH and PCs have a high affinity for metal binding at their thiol groups. PCs in the form of ( $\gamma$ -GluCys)*n*-Gly are a kind of polypeptide derived from GSH ( $\gamma$ -GluCysGly) (Cobbett and Goldsbrough 2002). PCs have been demonstrated to be important for heavy metal detoxification. PC synthase (PCS)-deficient mutants of Arabidopsis and *Schizosaccharomyces pombe* are highly sensitive to Cd and arsenate (Cobbett and Goldsbrough 2002). Thus, overexpression of PCS genes was expected to increase metal tolerance and accumulation in plants. However, the results were variable; overexpression of AtPCS under the CaMV (*Cauliflower mosaic virus*) 35S promoter in tobacco plants increased accumulation of Cd and As in the root (Zanella et al. 2016), but overexpression of OsPCS1 (encoded by Os05g0415200) in rice under the same promoter decreased As accumulation in the grain (Hayashi et al. 2017). The difference is most probably due to the effect of PC on the translocation of As through the vasculature. The reduction of As in the rice grain was most likely to be because OsPCS1 expressed in other tissues blocked As translocation to the grain, by facilitating OsABCC1-mediated As sequestration into the vacuole. Consistent with this explanation, the grain As content of the OsPCS1 loss-of-function mutant was >5 times higher than that of the wild-type plant, and the node As content was less than 1/10th that of the wild type. Recently, Uruguchi et al. (2017) found that OsPCS1 encoded by Os06g0102300 had contrasting effects on As and Cd accumulation in rice grain; the concentration of As increased in the T-DNA knockout line of OsPCS1, but the concentration of Cd decreased. The knockout mutant lines of OsPCS1 showed sensitivity to both As and Cd. These results indicate the importance of PC in the accumulation of As and Cd in rice grain (Hayashi et al. 2017, Uruguchi et al. 2017).

GSH is an important tripeptide that not only confers metal tolerance, but also functions as an antioxidant with crucial roles in redox signaling and plant growth and development (Noctor et al. 2012). In addition, GSH is the precursor of PCs (Cobbett and Goldsbrough 2002). GSH metabolism is thus important in metal tolerance and accumulation. Many genes confer metal tolerance through a GSH-dependent pathway. For example, overexpression of AtMAN3, which encodes an endo- $\beta$ -mannanase, led to enhanced Cd accumulation and tolerance via a GSH-dependent pathway (Chen et al. 2015). Treatment with mannose, a product of the enzyme, also increased Cd tolerance and accumulation in a GSH-dependent manner, whereas loss of function of MAN3 resulted in the opposite phenotypes. An increased mannose concentration led to an increase in PC content and the increased expression of genes in the GSH-dependent Cd tolerance pathway, including PCS1, PCS2, GR1 (*glutathione reductase 1*), GR2, ABCC1 and PDR8 (*pleiotropic drug resistance 8*). It remains to be resolved whether this

mannose-mediated increase in Cd tolerance is a physiological pathway that protects plants from Cd exposure *in vivo*, and which signaling pathway mannose uses to increase the expression of the many genes necessary for GSH-dependent Cd tolerance. In rice, OsCLT1, a CRT-like transporter, mediates the export of  $\gamma$ -glutamylcysteine and GSH from plastids to the cytoplasm, and thus is necessary for As and Cd accumulation and tolerance (Yang et al. 2016).

Arsenic has two major forms, As(V) and As(III), which are known as predominant As species in aerobic and anaerobic soils, respectively (Zhao et al. 2009). As(V) is readily reduced to As(III) by arsenate reductase within plant cells. Arsenate reductases have been demonstrated to be important for As tolerance and accumulation in both Arabidopsis and rice [AtHAC1 (High Arsenic Content 1)/ATQ1 (arsenate tolerance QTL1), OsHAC1;1, OsHAC1;2 and OsHAC4] (Chao et al. 2014, Sanchez-Bermejo et al. 2014, Shi et al. 2016, Xu et al. 2017). In arsenate reductase loss-of-function mutants, a decrease in the reduction of As(V) to As(III), the major form of As efflux at the root, results in increased translocation of As to the shoot, and causes growth retardation.

### Regulatory factors

Plant responses to heavy metal stress are likely to require precise regulatory mechanisms, at the level of transcription, post-transcription, translation and post-translation; however, these have not been intensively studied. Several transcription factors have been found to regulate Cd tolerance and accumulation. Heat shock transcription factor A4a (HsfA4a) confers resistance to Cd by up-regulating metallothionein gene expression in *Triticum aestivum* (wheat) and rice (Shim et al. 2009). ZAT6 (zinc finger of *Arabidopsis thaliana* 6), a C2H2-type zinc-finger protein (ZFP) family member, positively regulates Cd tolerance and accumulation by activating GSH-dependent PC synthesis genes (Chen et al. 2016). A novel GLABRA1 ENHANCER BINDING PROTEIN (GeBP) transcription factor, GPL4, controls the root avoidance of Cd in Arabidopsis (Khare et al. 2017). GPL4 facilitates the reorganization of root architecture to avoid root growth into Cd-containing soil, and instead to explore non-contaminated regions of the rhizosphere. The Cd content of *GPL4* mutants did not differ from that of their corresponding wild-type plants, indicating that *GPL4* does not directly regulate the expression of Cd transporters. Instead, mutation of *GPL4* led to differences in ROS (reactive oxygen species) concentrations and expression of genes involved in oxidative stress responses. MicroRNAs are major regulators at the post-transcriptional level. Several microRNAs, such as miR319, miR390, miR393 and miR398, were shown to regulate Cd tolerance by targeting different genes (Mendoza-Soto et al. 2012, Zhou et al. 2012, Ding et al. 2016).

Only a few studies have revealed the role of post-translational modification in heavy metal tolerance and accumulation. SnRK2 (SNF1-related protein kinase 2 protein) was found to regulate Cd tolerance by controlling ROS accumulation (Kulik et al. 2012). An inositol pentakisphosphate 2-kinase (AtIPK1) positively regulates As tolerance by controlling Pi homeostasis

(Sun et al. 2016). Recently, through site-directed mutagenesis screening of potential phosphorylation sites in AtABCC1, we found that phosphorylation of Ser846 is required for the As resistance function of AtABCC1 when expressed in PCS-expressing yeast (Zhang et al. 2017). Mutations of six other potential phosphorylation sites with alanine conferred reduced resistance and decreased the intracellular As content compared with wild-type AtABCC1 when expressed in the same yeast strain, suggesting that these mutations compromised the vacuolar sequestration of As by AtABCC1. Thus, phosphorylation may modulate the activity of AtABCC1 at multiple sites, but in planta studies are necessary to clarify this possibility. Overexpression of a rice heavy metal-induced RING E3-ligase (OsHIR1) in Arabidopsis conferred resistance to both As and Cd, and reduced their accumulation (Lim et al. 2014). However, the roles of OsHIR1 remain to be revealed *in vivo*.

### Potential Applications

#### Development of contamination-free crops

Toxic metal accumulation in crops, especially rice, which is a staple food for about one-third of the world's population, poses a serious threat to human health. Reducing toxic metal accumulation in the edible parts of crops is important for food safety and human health; thus, numerous agronomic efforts have aimed to achieve this goal (Clemens and Ma 2016). With the increasing knowledge of the molecular mechanisms underlying heavy metal accumulation, and the increased number of genes known to contribute to this process, genetic and molecular biological approaches seem to offer a promising approach to achieve this goal.

Many low Cd-accumulating rice alleles, harboring mutations in genes such as *HMA3*, *NRAMP5* and *LCT1* (*Low-affinity cation transporter 1*), have been identified using various approaches, including loss-of-function mutant screening, targeting induced local lesions in genomes (TILLING) populations and dissection of natural variation in metal accumulation (Ueno et al. 2010, Uruguchi et al. 2011, Ishikawa et al. 2012, Takahashi et al. 2012). Some studies aiming to reduce Cd using transgenic approaches have been successful. For example, overexpression of *OsHMA3* selectively reduced Cd concentrations in the rice grain by sequestering Cd in the vacuoles of the root (Sasaki et al. 2014). A transgenic approach has also been used for reducing As concentration in the rice grains. Overexpression of *ScACR3* in rice under the control of the CaMV 35S promoter could reduce 20–30% of As in the rice grains (Duan et al. 2012).

#### Phytoremediation

Knowledge of vacuolar transporters can be used to develop plants with a high capacity to accumulate metals, which can be used in phytoremediation efforts. Several studies have pioneered the expression of vacuolar metal transporters in different plant species to increase their metal tolerance and accumulation. In 2003, Song et al. (2003) introduced the *ScYCF1*, a vacuolar GSH–Cd transporter, into Arabidopsis plants, and the plants exhibited a dramatically enhanced tolerance and accumulation

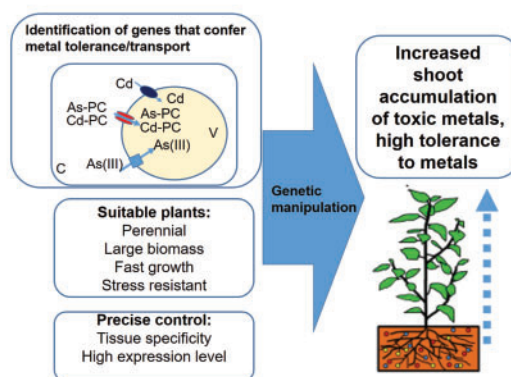
of Pb(II) and Cd(II). This study demonstrated the potential of vacuolar metal transporters for developing plants with an improved capacity for phytoremediation of heavy metal-polluted soils. However, most studies of this kind have been performed under laboratory conditions, which are very different from those in the field. Metals in soil are present in a variety of chemical forms and oxidation states, as well as in different complexed forms with organic compounds and matrix particles (Fasani et al. 2018). Nevertheless, due to regulatory restrictions on genetically modified organisms (GMOs), only a few studies have tested the phytoremediation efficiency of transgenic plants in the field (Yeagan et al. 1992, Bennett et al. 2003, Banuelos et al. 2005, Banuelos et al. 2007). Our group carried out the entire process of developing plants for phytoremediation (Shim et al. (2013)). We ectopically expressed *ScYCF1* in a non-flowering poplar, a plant that is ideally suited for phytoremediation, and tested the performance of the plant in mine tailing soil in a greenhouse, as well as under field conditions. The transgenic plants exhibited enhanced Cd tolerance and accumulation in both the laboratory and greenhouse trials. In the greenhouse trial, up to a 5-fold increase in Cd accumulation was observed in the shoots of the transgenic plants compared with the corresponding wild-type plants when grown in mine tailing soil. A field test was also carried out at an open site in Jinju, South Korea. Plants were grown in pots containing mine tailing soil. The transgenic plants produced a higher root biomass, but that of shoots was not altered. Only roots accumulated increased concentrations of metals, including Cd, Zn and Pb, indicating that the root to shoot translocation of toxic metals is one of the major limiting steps for removing metals from contaminated soil using these plants. The transgenic poplar plants might nevertheless be useful for stabilizing heavy metal-contaminated soils from erosion, since they could develop much more extensive root systems than the wild type in the mine tailing soil, and hence address an important problem in many contaminated regions. While this approach is promising, we are a long way from developing plants capable of extracting large amount of heavy metals for phytoremediation.

However, in the case of the metalloid As, it may not be long before genetic engineering produces plants capable of phytoremediation. Recently, Wang et al. (2018) converted *A. thaliana* into an As hyperaccumulator (accumulating 250 nmol g<sup>-1</sup> FW As in shoots) by heterologously expressing PvACR3 in the *athac1* background (Wang et al. 2018). These transgenic plants were even more effective accumulators of As in the shoot than were *P. vittata*, a fern renowned for its ability to hyperaccumulate As. AtHAC1 is an As reductase that reduces As(V) to As(III). Mutation in the As reductase decreased As efflux into the medium (Chao et al. 2014). Interestingly, the vacuolar As transporter ACR3, although strongly expressed in the roots of the transgenic plants, did not increase As(III) efflux to the external medium, nor did it sequester As into the vacuole, but instead helped load As into the vasculature and consequently promoted translocation to the shoot. Thus, generating plants that can clean up As-contaminated soil might be possible by manipulating the expression of these two genes in high biomass plants such as poplar.

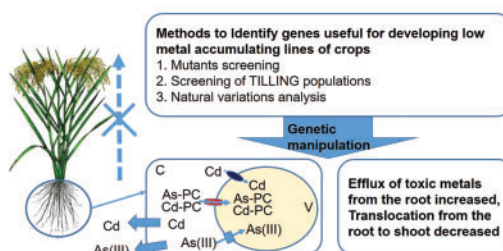
## Perspectives

Recent advances in the understanding of the mechanisms involved in plant metal accumulation, especially the identification of vacuolar metal transporters in various plant species, provide us with various tools to develop crops that are free of metal contamination or plants that hyperaccumulate metals for phytoremediation (Fig. 3). In addition to the vacuolar transporters, other transporters which export heavy metal-chelating compounds can be useful for the purpose. They include transporters of phenolics, carboxylates and

### A Strategy for developing high-capacity metal hyperaccumulators for phytoremediation



### B Strategy for developing contamination-free crops



**Fig. 3** Strategy for developing high-capacity metal hyperaccumulators for phytoremediation or contamination-free crops. (A) To develop hyperaccumulators, it is important to increase the metal accumulation in the shoot and to enhance the overall tolerance to metals. First, it is necessary to identify genes that confer tolerance to or transport of toxic metals, such as HMA3, ABCC1, ABCC2 and ACR3. Secondly, it is important to select a suitable background plant to introduce the genes for use in phytoremediation. The plant should have a large biomass, fast growth and stress resistance. Perennials might be ideal, since the root, once established, can be used for many years. Additionally, it is necessary to control the tissue-specific expression of the genes precisely to maximize the root to shoot translocation of the metals. High expression of the introduced gene is likely to be favorable. (B) In contrast, to develop contamination-free crops, it is important to reduce the toxic metals in the edible part, e.g. the rice grain. Multiple approaches are used to identify rice alleles with low toxic metal accumulation in grain, such as screening of loss-of-function mutants, targeting induced local lesions in genomes (TILLING) populations and natural variations. Then the genes identified are introduced together to increase efflux of metals from the root and to reduce root to shoot translocation in the case of rice.

nicotinamine, which can chelate and thereby extract heavy (toxic) metals from the soil. Also, overexpression of enzymes that produce such chelators will help increase vacuolar deposition of the toxic metals and metalloids. Although developing contamination-free crops and hyperaccumulators seem to be opposite to each other, similar tools we outline below can be used for both purposes.

1. Gene-stacking technique. By using gene-stacking technology, which involves introducing multiple genes that confer diverse new capabilities, including root uptake, root to shoot translocation and detoxification, it will be possible to improve the efficiency of contamination-free crops and hyperaccumulators. Important advances in developing hyperaccumulators will be possible if the translocation of metals from the root to the shoot is increased by decreasing the activity of vacuolar transporters in the root and, in the same plant, the tolerance of the shoot to the toxic metals is increased. Such a combinatory approach is important since increasing only the root to shoot translocation has an adverse effect (Hannikenne et al. 2008). For development of crops safe from toxic metal contamination in the grain, root sequestration should be increased to reduce root to shoot translocation of toxic metals and, here also, use of multiple genes may be able to increase the efficiency.
2. Controlling the expression of the introduced genes, including the expression pattern and expression levels. (1) In most cases, the transporters were introduced into plants under general overexpression promoters, as in the studies carried out by Song et al. (2003) and Shim et al. (2013). Genes driven by the 35S or ubiquitin promoters are expressed in many different tissues in plants. However, if vacuolar transporters are highly expressed specifically in the root tissues, large amounts of heavy metals can be sequestered in the root vacuoles instead of being translocated to the shoot, which would greatly reduce contamination of the shoot and grain. In contrast, using shoot mesophyll-specific promoters to drive the expression of vacuolar transporters might yield plants with an elevated capacity for phytoextraction. (2) Increasing the expression level of the introduced genes might further improve the efficiency of the transgenic plants. In heavy metal hyperaccumulators, the high copy number of major heavy metal transporters, such as HMA4 and HMA3, greatly increases the expression of these transporters, thus enhancing the ability to accumulate metals (Hannikenne et al. 2008, Ueno et al. 2011, Yan et al. 2016).
3. Controlling the substrate specificity of the transporters. Toxic heavy metals are transported by essential metal transporters. Overexpression of these transporters may alter the homeostasis of the essential metals, and consequently the productivity of the plant. However, some transporters exhibit a high substrate specificity, e.g. OsHMA3, NcHMA3 and SaHMA3 specifically transport Cd (Ueno et al. 2010, Ueno et al. 2011, J. Zhang et al. 2016). Deciphering the mechanisms that dictate the substrate specificity of the metal transporters would help to develop safe crops or hyperaccumulators, which could specifically exclude or accumulate toxic heavy metals, without interfering with metal homeostasis.

In conclusion, the biotechnological tools needed to develop crop plants that are free of contamination and plants with a highly efficient phytoremediation capacity are already available.

We expect to witness the development of such plants in the near future.

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### Disclosures

The authors have no conflicts of interest to declare.

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