Research

New Phytologist

Dissecting the components controlling root-to-shoot arsenic translocation in *Arabidopsis thaliana*

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Received: *6 January 2017*
Accepted: *26 July 2017*

New Phytologist (2018) **217:** 206–218
doi: 10.1111/nph.14761

Key words: *Arabidopsis thaliana*, arsenate reductase, arsenic, arsenic accumulation, arsenite efflux, *Pteris vittata*, tolerance.

Summary

• Arsenic (As) is an important environmental and food-chain toxin. We investigated the key components controlling As accumulation and tolerance in *Arabidopsis thaliana*.

• We tested the effects of different combinations of gene knockout, including arsenate reductase (*HAC1*), γ-glutamyl-cysteine synthetase (*γ-ECS*), phytochelatin synthase (*PCS1*) and phosphate effluxer (*PHO1*), and the heterologous expression of the As-hyperaccumulator *Pteris vittata* arsenite efflux (*PvACR3*), on As tolerance, accumulation, translocation and speciation in *A. thaliana*.

• Heterologous expression of *PvACR3* markedly increased As tolerance and root-to-shoot As translocation in *A. thaliana*, with PvACR3 being localized to the plasma membrane. Combining *PvACR3* expression with *HAC1* mutation led to As hyperaccumulation in the shoots, whereas combining *HAC1* and *PHO1* mutation decreased As accumulation. Mutants of *γ-ECS* and *PCS1* were hypersensitive to As and had higher root-to-shoot As translocation. Combining *γ-ECS* or *PCS1* with *HAC1* mutation did not alter As tolerance or accumulation beyond the levels observed in the single mutants.

• PvACR3 and HAC1 have large effects on root-to-shoot As translocation. Arsenic hyperaccumulation can be engineered in *A. thaliana* by knocking out the *HAC1* gene and expressing *PvACR3*. PvACR3 and HAC1 also affect As tolerance, but not to the extent of γ-ECS and PCS1.

Introduction

Arsenic (As) is a toxic metalloid widely distributed in the environment. The transfer of As from soil to the edible parts of crop plants is of great concern as dietary exposure to As can present a significant risk to human health (Meharg *et al.*, 2009; Zhao *et al.*, 2010). Arsenic is present in soil primarily as arsenate [As(V)] or arsenite [As(III)] depending on the prevailing redox conditions. These As species are taken up inadvertently by plant roots via phosphate transporters in the case of As(V) (Shin *et al.*, 2004; González *et al.*, 2005; Wang *et al.*, 2016) or silicic acid transporters (Ma *et al.*, 2008) and some aquaporin channels in the case of As(III) (Isayenkov & Maathuis, 2008; Kamiya *et al.*, 2009; Xu *et al.*, 2015). Although As(V) and As(III) are taken up readily by roots, their translocation from roots to shoots is limited in most plant species studied to date (Raab *et al.*, 2007; Zhao *et al.*, 2009). A small number of fern species are able to hyperaccumulate As in the above-ground parts (Ma *et al.*, 2001; Zhao *et al.*, 2002). These plants are characterized by an exceptionally high ability to transport As from roots to the above-ground tissues (Su *et al.*, 2008; Zhao *et al.*, 2009). Thus, the translocation of As from roots to shoots appears to be the bottleneck controlling As accumulation in the above-ground tissues, although the underlying mechanisms remain unclear.

One possible determinant of the As translocation efficiency is the capacity of As(V) reduction in the roots. Recent studies have identified a new class of As(V) reductases in plants, named HAC1 or ATQ1, that play an important role in controlling As accumulation in the shoots (Chao *et al.*, 2014; Sanchez-Bermejo *et al.*, 2014; Shi *et al.*, 2016; Xu *et al.*, 2017). In *Arabidopsis thaliana*, loss of function of *HAC1* leads to a *c.* 50-fold increase in As accumulation in the shoots during short-term As(V) feeding hydroponic experiments and a 10-fold increase in shoot As concentration after 5-wk of growth in a potting mix spiked with 7.5 mg kg^{-1} As(V) (Chao *et al.*, 2014). In rice, the *oshac1;1 oshac1;2* double mutant and *oshac4* single mutant showed 2.3- and three-fold, respectively, higher As concentrations in the shoots compared with the wild-type (WT) (Shi *et al.*, 2016; Xu *et al.*, 2017). There are several possible reasons for the observed

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effect of HACs on the root-to-shoot As translocation. First, HACs reduce As(V) to As(III) to allow the latter to be extruded to the external medium, thus decreasing the cellular As content in the roots available for xylem loading (Chao *et al.*, 2014; Shi *et al.*, 2016; Xu *et al.*, 2017). Second, decreased As(V) reduction in the roots of *HAC* mutants may allow As(V) to be loaded into the xylem efficiently via phosphate transporters, although this hypothesis has not been tested. Third, As(V) reduction catalysed by HACs allows As(III) to be complexed by thiol compounds and subsequently sequestered in the vacuoles in the roots (Song *et al.*, 2010, 2014), therefore decreasing the mobility of As. There is some evidence that the complexation of As(III) with phytochelatins (PCs) decreases As(III) mobility from roots to shoots in *A. thaliana*, with the shoot-to-root As concentration ratio in the PC mutant *cad1;3* and the glutathione (GSH) mutant *cad1;2* being 5–10-fold higher than in WT plants (Liu *et al.*, 2010). In the As hyperaccumulator *Pteris vittata*, As(V) remains the predominant As species in the roots after As(V) exposure, and there is also very little As(III)–thiol complexation (Zhao *et al.*, 2003; Zhang *et al.*, 2004; Pickering *et al.*, 2006); both of which may contribute to the high efficiency of As translocation in *P. vittata*.

Another key determinant of root-to-shoot As translocation is xylem loading. Both As(III) and As(V) are found in the xylem sap, although there is usually more As(III) than As(V), even when plants are exposed to As(V) (reviewed in Zhao *et al.*, 2009). As (V) may be loaded into the xylem via phosphate transporters, such as PHO1, but *pho1* mutants did not show decreased As accumulation in the shoots compared with WT plants (Quaghebeur & Rengel, 2004), suggesting that As(III) is the main As species loaded into the xylem in WT plants. As(III) can be loaded into the xylem via NIP3;1 and NIP7;1 in *A. thaliana* (Xu *et al.*, 2015; Lindsay & Maathuis, 2016). In rice, the silicic acid efflux transporter Lsi2 is also able to transport As(III) out of the cells towards the stele for xylem loading (Ma *et al.*, 2008). A high expression of Lsi2 and its polar localization on the proximal side of endodermal cells probably explain the relatively high translocation of As(III) in rice (Ma *et al.*, 2008). In *P. vittata*, As(III) was found to be the main form of As in the xylem sap (Su *et al.*, 2008). However, how As(III) is loaded into the xylem in this As hyperaccumulator is still unknown. Indriolo *et al.* (2010) identified an As(III) efflux transporter, PvACR3, that plays an important role in As(III) tolerance by transporting As(III) into the vacuoles in the gametophyte of *P. vittata*. Intriguingly, heterologous expression of *PvACR3* in *A. thaliana* increased As translocation from roots to shoots (Chen *et al.*, 2013). Moreover, PvACR3 was found to be localized in the plasma membrane in transgenic *A. thaliana*, suggesting a role of PvACR3 in mediating As(III) efflux for xylem loading in this heterologous system (Chen *et al.*, 2013). Heterologous expression of the yeast *ScACR3* in rice or *A. thaliana* was found to increase As(III) efflux to the external medium, but the effects on As distribution between roots and shoots were inconsistent (Ali *et al.*, 2012; Duan *et al.*, 2012).

In the present study, we tested the effects of different combinations of gene knockout and heterologous expression on As accumulation, especially the mobility of As during root-to-shoot translocation, and As tolerance in *A. thaliana*. The genes tested included the As(V) reductase (*HAC1*), γ-glutamyl-cysteine synthetase (*γ-ECS*), PC synthase (*PCS1*), phosphate effluxer (*PHO1*) and *PvACR3*. We first tested the effect of *PvACR3* expression in the WT or *hac1* mutant background. This was followed by experiments investigating whether PHO1 affects As accumulation in the shoots differently in the WT or *hac1* background. Finally, we tested the effect of reduced thiol synthesis caused by *γ-ECS* or *PCS1* mutation in combination with *HAC1* mutation. Our results show that *HAC1* mutation combined with *PvACR3* expression dramatically increases As accumulation in *A. thaliana* shoots.

Materials and Methods

Plant materials

The plant materials used included *Arabidopsis thaliana* L. Heynh. WT Columbia-0 (Col-0), *cad1-3* (PC-deficient mutant) and *cad2-1* (GSH-deficient mutant) (Howden *et al.*, 1995a,b), two T-DNA insertion knockout mutants of *HAC1* (GABI_868F11 and SM_3_38332 for *hac1-1* and *hac1-2*, respectively) (Chao *et al.*, 2014), and the *pho1-2* mutant (Delhaize & Randall, 1995). All single mutants are in the Col-0 background. Double mutants *cad2-1 hac1*, *cad1-3 hac1* and *pho1-2 hac1* were generated by crossing the respective single mutants. Homozygous double mutants were identified by PCR genotyping of the F_2 progeny (Supporting Information Fig. S1; Table S1).

To generate the *35S::PvACR3* construct, *PvACR3* (UniProt #FJ751631) was cloned into pCC0869, a pBI121-derived plant transformation vector containing the CaMV 35S promoter. The *PvACR3* gene was PCR amplified with the 5′ primer 5′-GCTC TAGAATGGAGAACTCAAGCG-3′ (*Xba*I) and the 3′ primer 5′-TCCCCCGGGCTAAACAGAAGGCCCCTTC-3′ (*Sma*I) using cDNA derived from arsenate-grown gametophytes of *P. vittata*, and the resulting PCR fragment was confirmed by sequencing. The resulting fragment was inserted into the *Xba*I and *Sma*I-linearized pCC0869 vector. *Arabidopsis thaliana* (Col-0) was transformed with the *35S::PvACR3* construct using *Agrobacterium* and the floral dip method (Clough & Bent, 1998). Homozygous lines were identified in the T_3 generation via segregation analysis.

Two approaches were used to generate *HAC1* mutation and *PvACR3* expression lines. First, a *PvACR3* expression line (E8) in the Col-0 background was crossed to *hac1-1* and *hac1-2*. Homozygous *hac1-1 PvACR3* and *hac1-2 PvACR3* were identified from F_2 progeny by PCR genotyping. Second, *PvACR3* was expressed in *hac1* directly. *PvACR3* was cloned into the *2X*35S promoter cassette of pMDC32 between *Asc*I and *Pac*I restriction sites by recombination of the primers 5′-CGGGCCCCCC CTCGAGGCGCGCCATGGAGAACTCAAGCGCGGAGC-3′ (*Asc*I) and 5′-CCGCTCTAGAACTAGTTAATTAACTAAA CAGAAGGCCCCTTCC-3′ (*Pac*I), using the ClonExpress™ II one-step cloning kit (Vazyme, Nanjing, China). The binary vector pMDC32-*PvACR3* was transformed into *Agrobacterium* strain GV3101 by the freeze–thaw method. The *Agrobacterium* culture was used to transform *A. thaliana hac1-2* by *Agrobacterium*-mediated floral dip transformation (Clough & Bent, 1998).

Homozygous lines were identified in the T_3 generation, and three lines with relatively high levels of *PvACR3* expression in the roots were selected.

Arsenic tolerance assays

Arabidopsis thaliana seeds were surface sterilized and sown on agar plates containing MS medium (½ MS salts, 2% sucrose, pH 5.6, solidified with 1% agar) amended with various concentrations of As(V) or As(III). Each line was replicated in five plates. All plates were placed at 4°C in the dark for 2 d to synchronize germination. The plates were then placed vertically in a growth chamber at 22°C with a 16 h : 8 h, light : dark regime. The root length and shoot fresh weight were determined after 2 wk of growth.

Arsenic uptake and speciation

Different lines of *A. thaliana* were grown hydroponically with 1/5 strength Hoagland nutrient solution (Liu *et al.*, 2010). Four-week-old plants were exposed to 5 μM As(V) or As(III) for 24 h, with four replicates for each line. Phosphate was withheld in the As(V) experiments to facilitate As(V) uptake. As(V) uptake and As(III) efflux were estimated by measuring the changes in As speciation in the nutrient solution, as described previously (Liu *et al.*, 2010). Roots and shoots were harvested for the analysis of As speciation and concentration.

PvACR3 hac1 and WT seeds were germinated on 1/2 MS medium without As for 1 wk. Seedlings were transplanted into an As-contaminated soil for 3 wk. The soil contained 101 mg kg^{-1} total As as a result of nearby mining activities. Each pot was filled with 0.3 kg of soil and planted with either four *A. thaliana* plants or one plant of *P. vittata* (two- to three-frond stage), with four replicates per line. *PvACR3 hac1* and WT plants were also grown in trays containing a vermiculite-based potting compost spiked with 10 mg kg^{-1} As(V), with five replicates per line. Plants were grown in a growth chamber at 22°C with a 16 h : 8 h, light : dark regime. *Arabidopsis thaliana* plants were harvested 3 wk after transplanting, whereas *P. vittata* plants were harvested 3 and 6 wk after transplanting.

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were extracted from 10-d-old *A. thaliana* roots and shoots using the RNeasy plant Mini Kit (BioTeKe, Beijing, China). Reverse transcription was carried out using the R233-01 kit (Vazyme). Semi-quantitative RT-PCR was performed in a reaction mixture of 20 μl of 2 × Taq Master Mix (Vazyme) for 30 cycles. *Actin2* was used as the reference gene (primers: forward, 5′-TCACAGCACTTGCACCAAGCA-3′; reverse, 5′-AACGATTCCTGGACCTGCCTCA-3′). *HAC1* and *PvACR3* were PCR amplified using the primer sets 5′-GAAGATG TTGAGACCGTTGATGTTT-3′ (forward) and 5′-TCACT TTCAAGTTTCAAGTGCCGAT-3′ (reverse) for *HAC1*, and 5′-ATGGAGAACTCAAGCGCG-3′ (forward) and 5′-GACCC CACCCAGCATTTCAT-3′ (reverse) for *PvACR3*.

Subcellular localization of PvACR3

To investigate the subcellular localization of PvACR3 expressed in *A. thaliana*, a *35S::PvACR3-GFP* fusion construct was generated. The *Aequorea coerulescens* green fluorescent protein (*AcGFP*) coding region was amplified from plasmid pUC-AcGFP and inserted into the *Sma*I and *Eco*RI sites of the plasmid pGreen 0299 plant transformation vector to make pGreen-*AcGFP* constructs. The full-length *PvACR3* gene was amplified from *P. vittata* cDNAs generated from RNA isolated from arsenate-treated gametophytes using the primers 5′-GCTCTAGAATG GAGAACTCAAGCG-3′ (*Xba*I, forward) and 5′-TCCCCCGG GAACAGAAGGCCCCTTCCTC-3′ (*Sma*I, reverse), and then cloned in frame to *AcGFP*. The final construct was confirmed by sequencing. The construct was introduced into *A. thaliana* (Col-0) by *Agrobacterium*-mediated transformation. Stably transformed *A. thaliana* plants expressing *PvACR3-GFP* were selected for GFP fluorescence analysis. Images were acquired using a Zeiss LSM 710 laser confocal microscope (Carl Zeiss Co., Jena, Germany). To label *A. thaliana* seedlings with the plasma membrane dye FM4-64 (T13320, Invitrogen), 5-d-old whole seedlings grown on Petri dishes were incubated with 1 μM FM4-64 in water for 15 min. The seedlings were rinsed in distilled water and imaged immediately. To further confirm the subcellular localization of PvACR3, total microsomal membrane fractions were isolated from 4-wk-old whole transgenic *A. thaliana* expressing *PvACR3*. Aqueous two-phase extractions were performed as described previously (Indriolo *et al.*, 2010).

Determination of As speciation and total As concentration

At the end of As exposure, roots were desorbed of apoplastic As in an ice-cold solution containing 1 mM K_2HPO_4, 0.5 mM Ca $(NO_3)_2$ and 5 mM MES (pH 5.5) for 10 min (Xu *et al.*, 2007). Roots and shoots were rinsed with deionized water, blotted dry and weighed. Plant samples were ground in liquid nitrogen to a fine powder. Subsamples (*c.* 0.1 g) of the ground materials were extracted with 10 ml of a phosphate buffer solution (2 mM NaH_2PO_4, 0.2 mM Na_2-EDTA, pH 5.5). Arsenic species in the nutrient solution and in the root and shoot extracts were determined using high-performance liquid chromatography linked to inductively coupled plasma mass spectrometry (HPLC-ICP-MS; NexIon 300x, Perkin-Elmer, Waltham, MA, USA), as described previously (Xu *et al.*, 2007). For the determination of total As concentration in plant samples, plant tissues were washed with deionized water and dried at 65°C for 2 d. Dried plant samples were digested with 5 ml mix acids of HNO_3/$HClO_4$ (85 : 15 v/v) in a digestion block. The digests were diluted with 2% HNO_3 and As concentrations were determined using ICP-MS (Perkin Elmer NexIon 300x).

Determination of nonprotein thiols

After 24 h exposure to As(V), *A. thaliana* roots and shoots were separated and immediately frozen in liquid nitrogen. Nonprotein thiol compounds (cysteine (Cys), GSH, PC_2, PC_3 and PC_4) were

extracted and quantified using an HPLC method with monobromobimance (mBBr) derivatization, as described previously (Minocha *et al.*, 2008).

Statistical analysis

One-way or two-way analysis of variance (ANOVA) was performed to test the significance of treatment effects, followed by comparisons of treatment means using Tukey's honestly significant difference (HSD) test. Data were transformed logarithmically before ANOVA to stabilize the variance where necessary. For the As tolerance assays, the dose–response data were fitted to a log-logistic equation to estimate the effect concentration causing 50% inhibition (EC_{50}).

Results

Heterologous expression of *PvACR3* enhances As tolerance and accumulation in *A. thaliana*

Two independent lines of transgenic *A. thaliana* (Col-0) expressing *PvACR3* (E4 and E8) were selected for As(V) and As(III) tolerance assays (Fig. S2). In the absence of As(III), no significant differences in root growth or shoot fresh weight were observed between WT and transgenic lines (Fig. S2c,d). In the presence of toxic As(III) concentrations (25–100 μM As(III)), both lines grew significantly better than WT plants (Fig. S2b–d). The EC_{50} values were estimated from the fitted dose–response curves (Table 1). Compared with WT, the expression of *PvACR3* increased the EC_{50} of As(III) by approximately two- to three-fold. The two *PvACR3* transgenic lines also displayed enhanced tolerance to As(V) compared with WT plants (Fig. S2b,e,f). The As(V) EC_{50} values for the two *PvACR3* transgenic lines were 4.0 to 5.8 times those for WT based on root growth, and 2.1 to 2.6 times those for WT based on shoot biomass (Table 1). These results demonstrate that the expression of *PvACR3* confers both As(III) and As(V) tolerance in *A. thaliana*.

We next investigated the effect of *PvACR3* expression on As accumulation in *A. thaliana*. Plants were grown hydroponically and then exposed to 5 μM As(III) or As(V) for 2 d. In the As(III) experiment, the two *PvACR3* transgenic lines accumulated significantly lower concentrations of As (by 35–45%) in the roots than WT plants, but accumulated 25- to 34-fold higher As concentrations in the shoots (Fig. 1a,b). The ratio of the shoot-to-root As concentration was 0.12–0.20 in the transgenic lines, compared with 0.003 in WT plants. Similar results were obtained in the As (V) experiment, with the transgenic lines accumulating 28–50% lower total As in the roots, but 29–37-fold higher total As in the shoots than WT plants (Fig. 1c,d). The shoot-to-root As concentration ratio was 0.3–0.6 in the transgenic lines, compared with 0.008 in WT plants. Arsenic speciation in root and shoot tissues was determined in the As(V) experiment. As(III) was the predominant As species in WT plants, accounting for 92% and 94% of the total As in the roots and shoots, respectively, indicating an efficient As(V) reduction in *A. thaliana*. The expression of *PvACR3* decreased the percentage of As(III) in the roots to 88–89%, but increased the percentage of As(III) in the shoots to 95–96%.

Because *PvACR3* encodes an As(III) efflux transporter (Indriolo *et al.*, 2010), we also estimated As(III) efflux from roots to the external medium following As(V) uptake in the As(V) exposure experiment. We found no significant differences between WT and *PvACR3* transgenic plants in either As(V) uptake or As (III) efflux, with As(III) efflux accounting for 82–85% of the As (V) uptake after 1 d of As(V) exposure (Fig. 1e).

To determine whether the expression of *PvACR3* in the root, shoot or both is important in conferring As tolerance in *A. thaliana*, four types of reciprocal grafts between WT (Col-0)

Table 1 The effect concentration of arsenite (As(III)) or arsenate (As(V)) causing 50% inhibition of root or shoot growth ($EC_{50} \pm SE$) of Col-0, mutants and *PvACR3* expression lines of *Arabidopsis thaliana*

		As(V) EC_{50} (μM)		As(III) EC_{50} (μM)	
	Lines	Root length	Shoot biomass	Root length	Shoot biomass
Exp 1[a]	Col-0	76.5 ± 15.4	88.9 ± 155.9	12.9 ± 3.4	17.2 ± 8.9
	PvACR3-E4	307.4 ± 56.3	230.0 ± 58.3	29.6 ± 5.0	36.9 ± 7.5
	PvACR3-E8	444.0 ± 36.1	185.6 ± 81.5	38.9 ± 4.1	42.0 ± 17.6
Exp 2[a]	Col-0	243.6 ± 14.3	106.9 ± 10.3		
	hac1-2	90.9 ± 17.7	93.8 ± 21.7		
	PvACR3-E8 *hac1-2*	222.4 ± 55.4	253.4 ± 80.4		
Exp 3[a]	Col-0	>250[b]	163.3 ± 34.3		
	cad2-1	21.8 ± 1.0	8.2 ± 1.4		
	hac1-2	92.8 ± 11.5	94.8 ± 11.1		
	cad2-1 hac1-2	19.2 ± 1.3	7.8 ± 1.6		
Exp 4[a]	Col-0	>250[b]	208.7 ± 33.5		
	cad1-3	23.2 ± 1.4	8.4 ± 1.4		
	hac1-2	147.9 ± 31.9	136.8 ± 18.4		
	cad1-3 hac1-2	24.3 ± 10.3	11.4 ± 1.4		

[a]Exps 1 and 2–4 used different agars, which contained different phosphate concentrations and therefore resulted in different EC_{50} values for Col-0.
[b]Where the highest As dose did not result in significant inhibition, EC_{50} could not be estimated and was considered to be higher than the largest As concentration in the experiment.

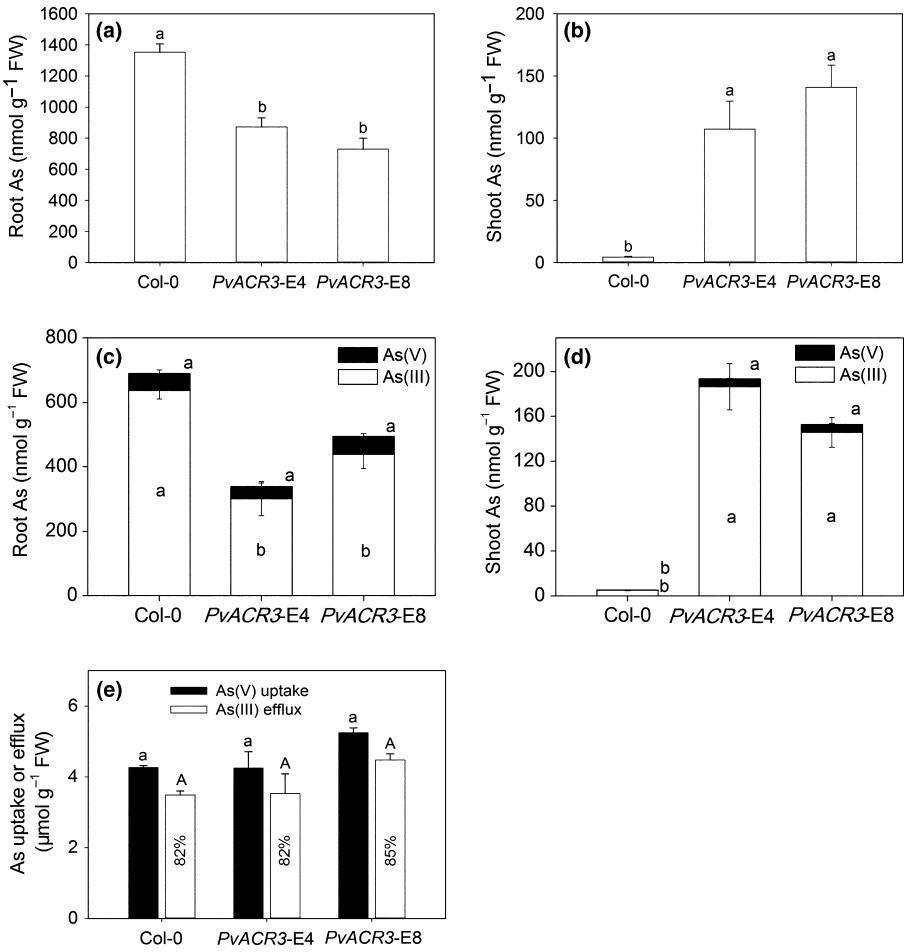

Fig. 1 Arsenic (As) accumulation in transgenic *Arabidopsis thaliana* expressing *PvACR3*. (a, b) Total As concentration in the roots (a) and shoots (b) of Col-0 and *PvACR3*-expressing lines after plants had been exposed to 5 μM arsenite (As(III)) for 2 d. (c, d) Concentrations of As(III) and arsenate (As(V)) in the roots (c) and shoots (d) of Col-0 and *PvACR3*-expressing lines after plants had been exposed to 5 μM As(V) for 2 d in the absence of phosphate. (e) As(V) uptake and As(III) efflux to the external solution after plants had been exposed to 5 μM As(V) for 1 d in the absence of phosphate. Data are the means ± SE ($n = 4$). The percentage values in (e) represent As(III) efflux as a percentage of As(V) uptake. Different letters above the bars indicate significant differences at $P < 0.05$. FW, fresh weight.

and *PvACR3*-E8 plants were generated. No significant differences in root growth or shoot fresh weight between the four different graft types grown in the absence of As(III) were observed (Fig. 2). Self-grafted WT plants showed decreased root growth and shoot fresh weight in the presence of 25 μM As(III), whereas self-grafted *PvACR3*-E8 plants did not. When WT shoot scions were grafted to *PvACR3*-E8 rootstocks, the resulting plants showed As tolerance comparable with that of self-grafted *PvACR3*-E8 plants grown in the presence of 25 μM As(III) (Fig. 2). However, when *PvACR3*-E8 shoot scions were grafted to WT rootstocks, the root growth and shoot fresh weight of the resulting plants were similar to those of self-grafted Col-0 plants grown in the presence of As (III). This experiment demonstrates that As tolerance in *PvACR3*-expressing plants is driven by the expression of *PvACR3* in the root and not the shoot.

PvACR3 is localized to the plasma membrane in transgenic *A. thaliana* plants

The subcellular localization of the PvACR3 protein in *A. thaliana* was determined by expressing *PvACR3:AcGFP* under the control of the CaMV 35S promoter. Leaves and roots from four independent transformed lines were incubated in the plasma membrane dye FM4-64. Green fluorescence from AcGFP was observed to co-localize with the red fluorescence of FM4-64 in both leaves and roots (Fig. S3a), indicating that the PvACR3:AcGFP fusion protein localizes to the plasma membrane. Furthermore, *PvACR3:AcGFP* appears to be preferentially expressed in the endodermis and the stele of the transgenic *A. thaliana* roots (Fig. S4).

The plasma membrane localization of PvACR3:AcGFP was also confirmed by protein immunoblotting using antibodies to GFP after membrane purification using two-phase extraction (Fig. S3b). Membranes collected in the upper phase of the extraction system are enriched in plasma membrane relative to the lower phase, as shown by the enrichment of the P-type ATPase plasma membrane marker and the depletion of the V-type ATPase vacuolar membrane marker in the upper phase. The plasma membrane-enriched upper phase is also enriched in AcGFP, confirming the plasma membrane localization of the ACR3:AcGFP fusion protein.

Combining *HAC1* mutation with *PvACR3* expression leads to As hyperaccumulation in *A. thaliana*

Because mutation of *HAC1* also results in a large increase in As accumulation in the shoots (Chao *et al.*, 2014), we tested the combined effect of *HAC1* mutation with *PvACR3* expression. We first crossed *PvACR3*-E8 with two T-DNA insertion *HAC1* knockout lines and obtained homozygous lines combining

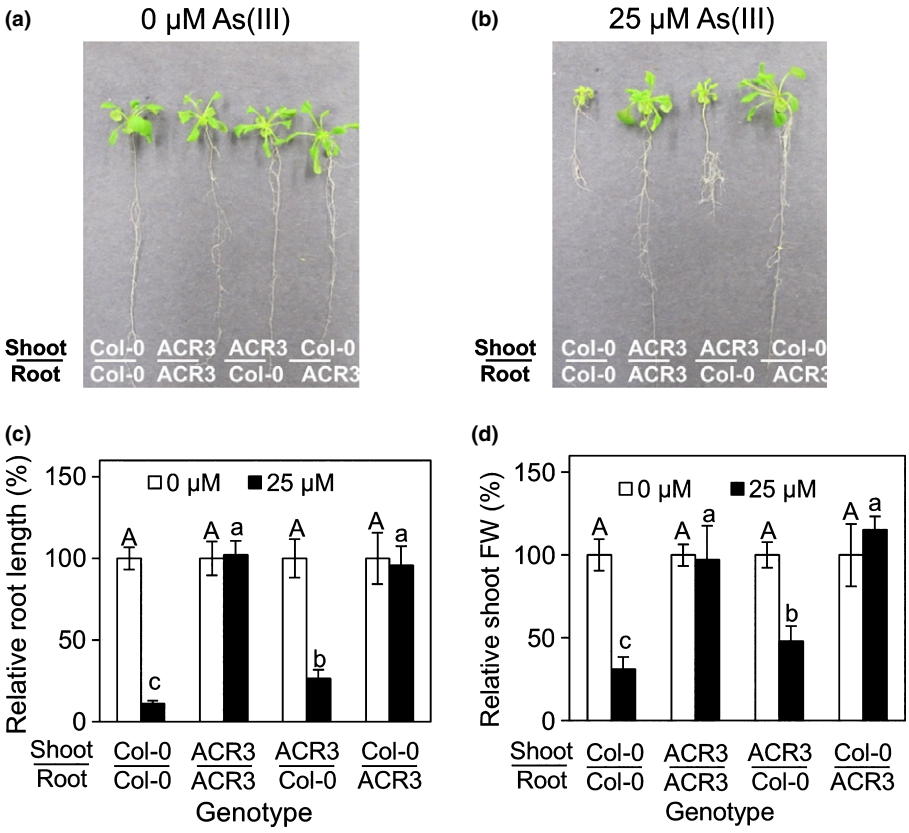

Fig. 2 Arsenic (As) tolerance of reciprocally grafted *Arabidopsis thaliana* plants expressing *PvACR3*. Reciprocally grafted seedlings were grown on plates in the absence (a) or presence (b) of 25 μM arsenite (As(III)); self-grafted Col-0 (Col-0/Col-0); self-grafted *PvACR3*-expressing line (ACR3/ACR3); *PvACR3*-expressing line shoot scion with Col-0 rootstock (ACR3/Col-0); Col-0 shoot scion with *PvACR3*-expressing line rootstock (Col-0/ACR3). (c) Root growth and (d) shoot fresh weight (FW) in the presence of 25 μM arsenite relative to control treatment. Data points are the mean ± SE ($n = 4$). The same letter within the graphs represents lines that are not significantly different ($P > 0.05$). The *PvACR3*-E8 line was used as a *PvACR3*-expressing transgenic line.

PvACR3 expression with *HAC1* mutation. The As(V) tolerance results of *PvACR3*-E8 *hac1-1* and *PvACR3*-E8 *hac1-2* are similar, so that only the dataset of the former is shown here. Consistent with the results shown in Fig. S2 and those reported by Chao *et al.* (2014), *PvACR3* expression increased As(V) tolerance in *A. thaliana*, whereas *hac1* mutants were more sensitive to As(V) than WT plants (Fig. S5; Table 1). In this experiment, the highest As(V) concentration (250 μM) did not significantly inhibit the root or shoot growth of *PvACR3*-E8; therefore, the exact EC_{50} could not be estimated (Table 1). The expression of *PvACR3* in *hac1* mutants enhanced the As(V) tolerance to a level that was similar to or higher than that of WT plants, but lower than that of *PvACR3*-E8 plants, especially at the high (250 μM) As(V) concentration (Fig. S5; Table 1).

We then determined As accumulation and speciation in hydroponically grown plants exposed to 5 μM As(V) for 1 d. *HAC1* mutation resulted in a 35% decrease in the root As(III) concentration, but a 16- to 24-fold increase in the root As(V) concentration, compared with WT (Fig. 3a). The percentage of As(III) in the root (relative to total As) decreased from 90% in WT plants to 19–27% in *hac1* mutants, which is indicative of a loss of function of a key As(V) reductase. *PvACR3* expression in the Col-0 background decreased the As(III) concentration in the roots by 74%, but had little effect on the As(V) concentration. Combining *PvACR3* expression with *HAC1* mutation decreased both As (III) and As(V) concentrations in the roots compared with the *hac1* mutants. *HAC1* mutation, *PvACR3* expression and the combination of the two genetic events produced striking phenotypes in As accumulation in *A. thaliana* shoots. Compared with WT (Col-0), *HAC1* mutation and *PvACR3* expression increased shoot As concentration by 18–19- and 58-fold, respectively, whereas combining the two events increased shoot As concentration by 114- to 117-fold (Fig. 3b). The increase in the shoot As concentration in *PvACR3*-E8 *hac1* plants was greater than the additive effect of *PvACR3*-E8 and *hac1* alone. In all lines, As(III) was the predominant As species in the shoots (> 90-%). In this experiment, the shoot-to-root As concentration ratio increased from 0.002 in WT to *c.* 0.015 in *hac1* mutants and 0.24 in *PvACR3*-E8 *hac1* plants. We also determined As(III) efflux from the roots to the external medium following As(V) uptake. In agreement with a previous study (Chao *et al.*, 2014), *HAC1* mutation greatly decreased As(III) efflux to the external medium (Fig. 3c). By contrast, *PvACR3* expression had little effect on this process in either the Col-0 or *hac1* background.

We also generated *PvACR3* expression lines in the *hac1* mutant (*hac1-2*) directly by transgenesis. Three independent lines of *PvACR3 hac1-2* (E6, E7, E11) were selected for further experiments (Fig. S6a). The three transgenic lines, *hac1-2* and Col-0 were grown for 3 wk in a vermiculite-based potting medium amended with or without 10 mg kg^{-1} As(V) (Fig. S6b). The addition of 10 mg kg^{-1} As(V) inhibited the shoot fresh weight of Col-0 and *hac1-2* by 28% and 41%, respectively. The inhibition on the three lines of *PvACR3 hac1-2* ranged from 31% to 52%, which was not significantly different from that in *hac1-2* (Fig. 4a). Compared with Col-0, *hac1-2* contained 8.5-fold higher total As concentration in the shoots (Fig. 4b). The

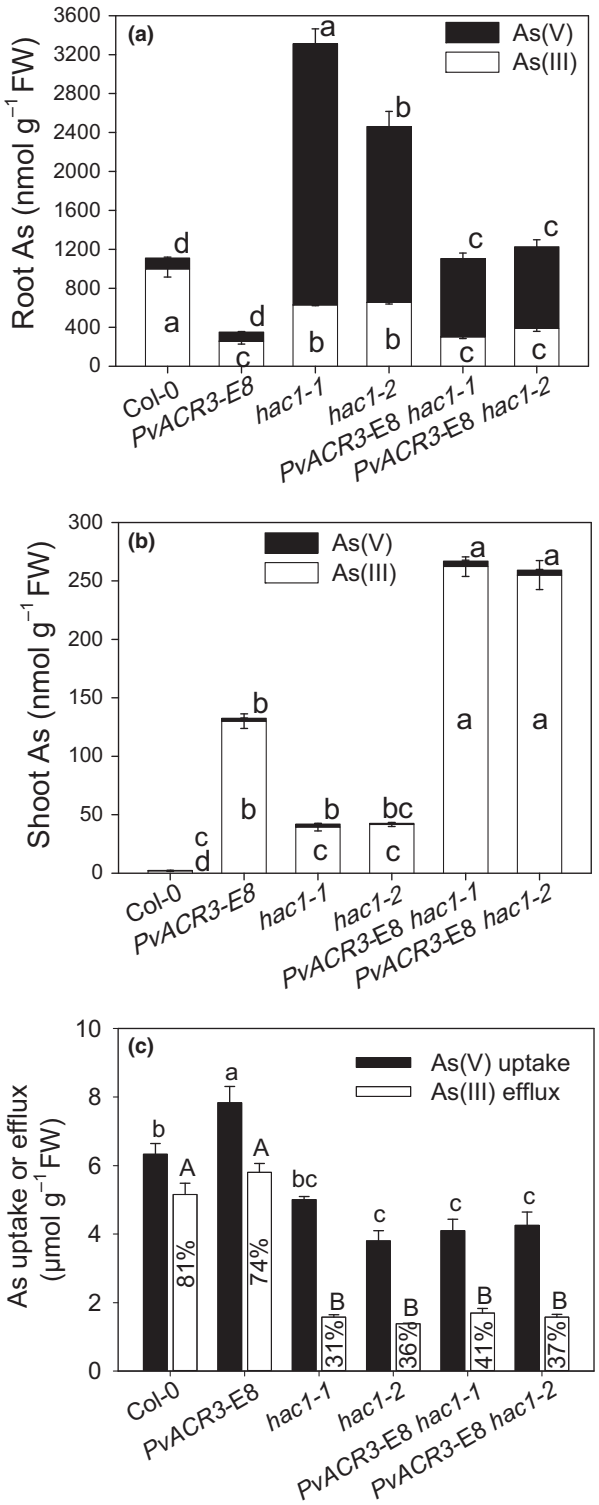

Fig. 3 Effect of combining *PvACR3* expression with *HAC1* mutation on arsenic (As) accumulation and speciation in *Arabidopsis thaliana*. (a, b) Arsenic speciation in the roots (a) and shoots (b) of different *A. thaliana* lines grown in hydroponic culture and exposed to 5 μM arsenate (As(V)) for 1 d without phosphate. (c) As(V) uptake and arsenite (As(III)) efflux into the nutrient solution after 1 d of exposure to 5 μM As(V) without phosphate. Data are the means ± SE ($n = 4$). The percentage values in (c) represent As (III) efflux as a percentage of As(V) uptake. Different letters above the bars indicate significant differences at $P < 0.05$. FW, fresh weight.

expression of *PvACR3* in *hac1-2* enhanced As accumulation in the shoots much more strongly, to 17–28 times of that in Col-0. The bioaccumulation factors (shoot-to-soil As concentration ratio) were 0.2, 2.0 and 4.0–6.1 in Col-0, *hac1-2* and *PvACR3 hac1-2*, respectively.

To assess the As accumulation ability of *PvACR3 hac1-2* plants in soil, the three transgenic lines, *hac1-2*, Col-0 and *P. vittata* were grown in an As-contaminated soil. The shoot biomass after 3 wk of growth was not significantly different between the different *A. thaliana* lines (Fig. 5a). Compared with Col-0, the shoot As concentration was 9.7 and 35–39 times higher in *hac1-2* and the three lines of *PvACR3 hac1-2*, respectively (Fig. 5b). After *P. vittata* was transplanted into the same soil for 3 or 6 wk, there was no significant increase in the frond biomass (Fig. 5c). There was also no significant increase in As concentration in the fronds 3 wk after transplanting compared with the initial As concentration at the time of transplanting (Fig. 5d). Six weeks after transplanting, the As concentration in *P. vittata* fronds increased from the initial level of 14 to 54 mg kg^{-1} (Fig. 5d). The net increase in frond As concentration was comparable with the shoot As concentrations of the *PvACR3 hac1-2* lines grown in the same soil over 3 wk.

Combining *HAC1* and *PHO1* mutation decreases As accumulation in *A. thaliana* shoots

Because *hac1* mutants accumulated large amounts of As(V) in the roots (Fig. 3a), we hypothesized that As(V) may be loaded into the xylem in the roots via the PHO1 phosphate exporter for long-distance transport to the shoots. To test this hypothesis, we crossed the *pho1-2* mutant (Delhaize & Randall, 1995) with the *hac1-1* mutant (Chao *et al.*, 2014) to generate a double mutant. In hydroponic culture with a normal level of phosphate concentration (0.1 mM), the *pho1-2* mutant plants were smaller than WT, with *c.* 70% and 40% inhibition of the shoot and root biomass, respectively (Fig. 6a). This phenotype is similar to that reported previously (Delhaize & Randall, 1995; Rouached *et al.*, 2011). The *hac1-1* mutant was also smaller than WT (by *c.* 20% in both the shoot and root biomass). The *hac1-1 pho1-2* double mutant showed the same growth phenotype as *pho1-2* (Fig. 6a). Arsenic speciation in the roots and shoots was determined after plants had been exposed to 5 μM As(V) for 1 d in the absence of phosphate. The *pho1*-2 mutant showed no significant differences from Col-0 in the total concentrations of As in the roots and shoots, and there were also no significant differences in As speciation, with As(III) accounting for 95–98% of the total As (Fig. 6b, c). By contrast, the *hac1-1* mutant showed 2.2 and 57 times higher As concentration in the roots and shoots, respectively, compared with Col-0. The increased root As concentration in *hac1-1* was mainly in the form of As(V), accounting for 58% of the total As, whereas most of the increased shoot As concentration was in the form of As(III). The As accumulation phenotype in *hac1-1* was reduced by 85% in the *hac1-1 pho1-2* double mutant, although the total As concentrations in the roots and shoots of the double mutant were still significantly higher than those of Col-0 (by 20% and six-fold, respectively) (Fig. 6b,c).

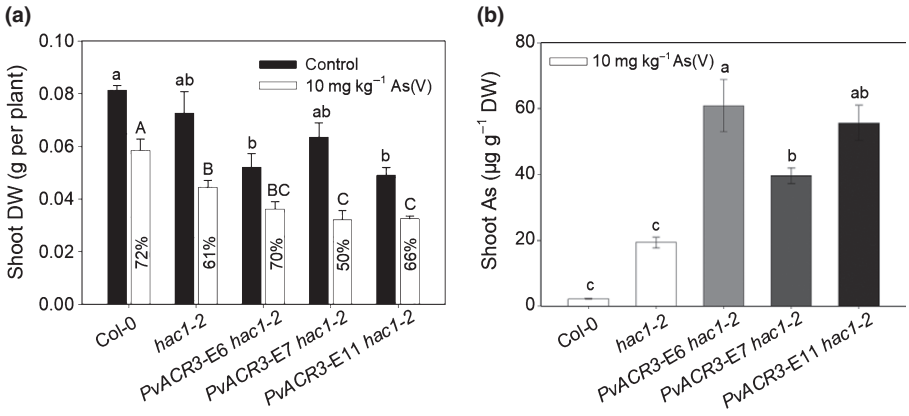

Fig. 4 Arsenic (As) accumulation in transgenic *Arabidopsis thaliana hac1* mutant expressing PvACR3 grown in a potting medium. (a) Shoot dry weights (DW) of different lines as affected by the addition of 10 mg kg^{-1} arsenate (As(V)). (b) Total As concentration in the shoots of different lines grown in a potting medium amended with 10 mg kg^{-1} As(V). Data are the means ± SE ($n = 4$). The values inside the open bars represent the percentage values relative to the control. Different letters above the bars represent significant differences at $P < 0.05$.

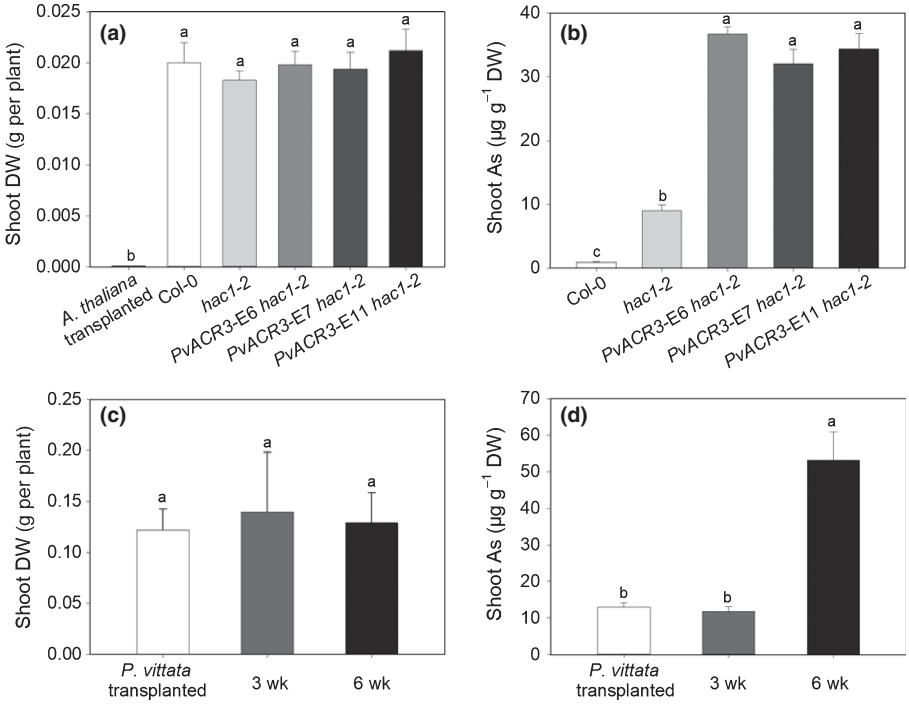

Fig. 5 Arsenic (As) accumulation in transgenic *Arabidopsis thaliana hac1-2* mutant expressing *PvACR3* and *Pteris vittata* grown in an As-contaminated soil. (a) Shoot dry weights (DW) of different *A. thaliana* lines. (b) Total As concentrations in the shoots of different lines of *A. thaliana* 3 wk after transplanting. (c) Frond dry weights of *P. vittata*. (d) Total As concentrations in the fronds of *P. vittata* at the time of transplanting and 3 or 6 wk after transplanting. Data are the means ± SE ($n = 4$). Different letters above the bars represent significant differences at $P < 0.05$.

The percentage of As(III) in the roots of the double mutant was 59%, which was lower than Col-0 and *pho1-2* (95–96%), but higher than *hac1-2* (42%). The results suggest that the *PHO1* mutation had no impact on As accumulation in the Col-0 background, but greatly suppressed As translocation from the roots to the shoots in the *hac1-2* background.

Effects of GSH and PC mutants in combination with the HAC1 mutation on As tolerance and accumulation

It is well known that GSH and PCs are crucial for As detoxification through the formation of As(III)–thiol complexes (Ha *et al.*, 1999; Pickering *et al.*, 2000; Liu *et al.*, 2010). Moreover, the sequestration of the As(III)–thiol complexes in the root vacuoles reduces the root-to-shoot translocation of As in *A. thaliana* Col-0 (Liu *et al.*, 2010). Here, we investigated the effects of combining mutations that reduce GSH (*cad2-1*) or PC (*cad1-3*) concentrations (Howden *et al.*, 1995a,b; Cobbett *et al.*, 1998) with *hac1-1* and *hac1-2* mutants (Chao *et al.*, 2014) on As tolerance and accumulation. As the double mutants of *cad2-1 hac1-1* and *cad1-3 hac1-1* behaved similarly to *cad2-1 hac1-2* and *cad1-3 hac1-2*, respectively, only one set of data is presented. In addition to genotyping based on the mutated genes, we also determined the concentrations of Cys, GSH and PCs in the roots and shoots of WT, single and double mutants after exposure to 5 μM As(V) for 1 d. The results are consistent with expectations, with *cad2-1* and *cad2-1 hac1-2* containing lower levels of GSH and PCs than WT, and *cad1-3* and *cad1-3 hac1-2* containing almost no PCs (Fig. S7).

Tolerance to As(V) was assessed by measuring the root and shoot biomass after plants had been grown on agar plates amended with 0–250 μM As(V). Consistent with previous reports (Ha *et al.*, 1999; Liu *et al.*, 2010), *cad2-1* and *cad1-3* mutants were hypersensitive to As(V), with EC_{50} being at least

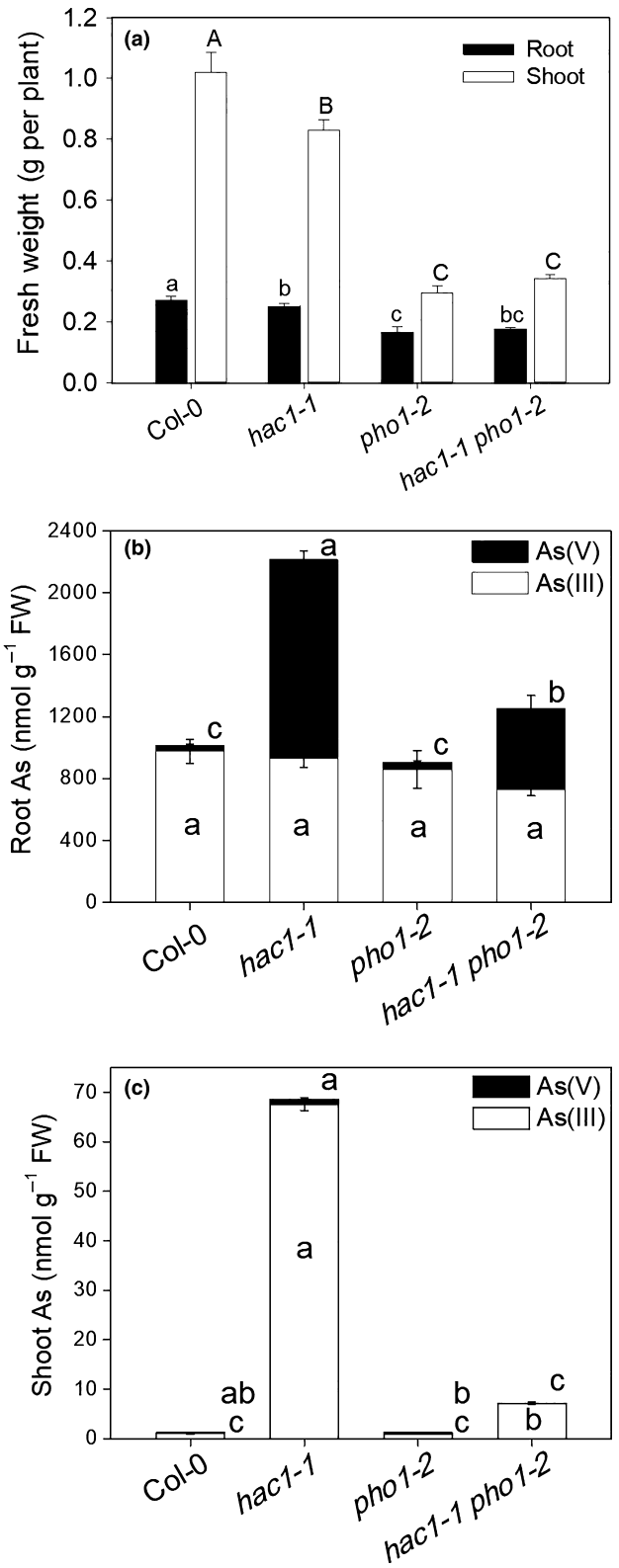

Fig. 6 Effect of combining *PHO1* and *HAC1* mutation on arsenic (As) accumulation in *Arabidopsis thaliana*. (a) Shoot and root fresh weights (FW) of Col-0, *hac1-1*, *pho1-2* and *hac1-1 pho1-2* plants of *A. thaliana*. (b, c) Arsenic speciation in the roots (b) and shoots (c) of different *A. thaliana* lines grown in hydroponic culture and exposed to 5 μM As(V) for 1 d without phosphate. Data are the means ± SE ($n = 4$). Different letters above the bars indicate significant differences at $P < 0.05$.

10 times lower than that of WT based on root growth and *c.* 20 times lower than that of WT based on shoot growth (Fig. S8; Table 1). *hac1* mutants were also more sensitive than Col-0, but not to the extent of *cad2-1* and *cad1-3* mutants. The *cad2-1 hac1-2* and *cad1-3 hac1-2* double mutants showed the same As (V) sensitivity as the *cad2-1* and *cad1-3* single mutants, respectively, with similar EC_{50} values between the single and double mutants (Table 1).

After exposure to 5 μM As(V) for 1 d, *hac1-2* accumulated two- and 12-fold more As in the roots and shoots, respectively, than Col-0 (Fig. 7a,b). The *cad2-1* and *cad1-3* mutants accumulated less As in the roots (*c.* 50%), but more As in the shoots (approximately three-fold) than Col-0. The *cad2-1 hac1-2* and *cad1-3 hac1-2* double mutants behaved similarly to the *hac1-2* single mutant, with the exception that *cad1-3 hac1-2* showed 25% lower shoot As concentration than *hac1-2*. With regard to As speciation, *HAC1* mutation markedly decreased the ability of roots to reduce As(V) to As(III), resulting in a decrease in the proportion of As(III) in the total As, from 85% in Col-0 to 18% in *hac1-2* (Fig. 7a). *cad2-1* and *cad1-3* mutants also exhibited a lower percentage of As(III) (76% and 80%, respectively) in the roots than WT. Combining either *cad2-1* or *cad1-3* with *hac1-2* further decreased the As(V) reduction ability, decreasing the percentage of As(III) in the *cad2-1 hac1-2* and *cad1-3 hac1-2* roots to 1.7% and 14%, respectively. Most of the As in the shoots was in the form of As(III) (93–100%), with little difference between Col-0, single and double mutants (Fig. 7b).

Discussion

Combining *HAC1* mutation with *PvACR3* expression leads to As hyperaccumulation in *A. thaliana*

Previous studies have shown that the *HAC1* mutation (Chao *et al.*, 2014) or the expression of *PvACR3* in the Col-0 background of *A. thaliana* (Chen *et al.*, 2013) markedly increases As accumulation in the aboveground tissues of *A. thaliana*. Here, we show that a combination of these two genetic events leads to As hyperaccumulation in the shoots of *A. thaliana* (Figs 3–5). The combined effect is more than the additive effect of the two events alone. Moreover, when *hac1 PvACR3* plants were grown in an As-contaminated soil for 3 wk, they accumulated As in the shoots to levels comparable with the As-hyperaccumulating fern *P. vittata* grown on the same soil for 6 wk after transplanting (Fig. 5).

Consistent with a previous study (Chao *et al.*, 2014), *HAC1* mutation leads to a greatly decreased As(III) efflux from the roots to the external medium following As(V) uptake and a markedly increased As translocation from the roots to the shoots (Fig. 3). This effect has also been observed in the rice mutants of *OsHAC1;1*, *OsHAC1;2* and *OsHAC4*, which are homologous genes of *HAC1* (Shi *et al.*, 2016; Xu *et al.*, 2017). By contrast, expression of the *P. vittata* As(III) efflux transporter gene *PvACR3* in *A. thaliana* did not increase the efflux of As(III) to the external medium (Figs 1, 3). This result is different from that of the study of Chen *et al.* (2013), which reported an increased

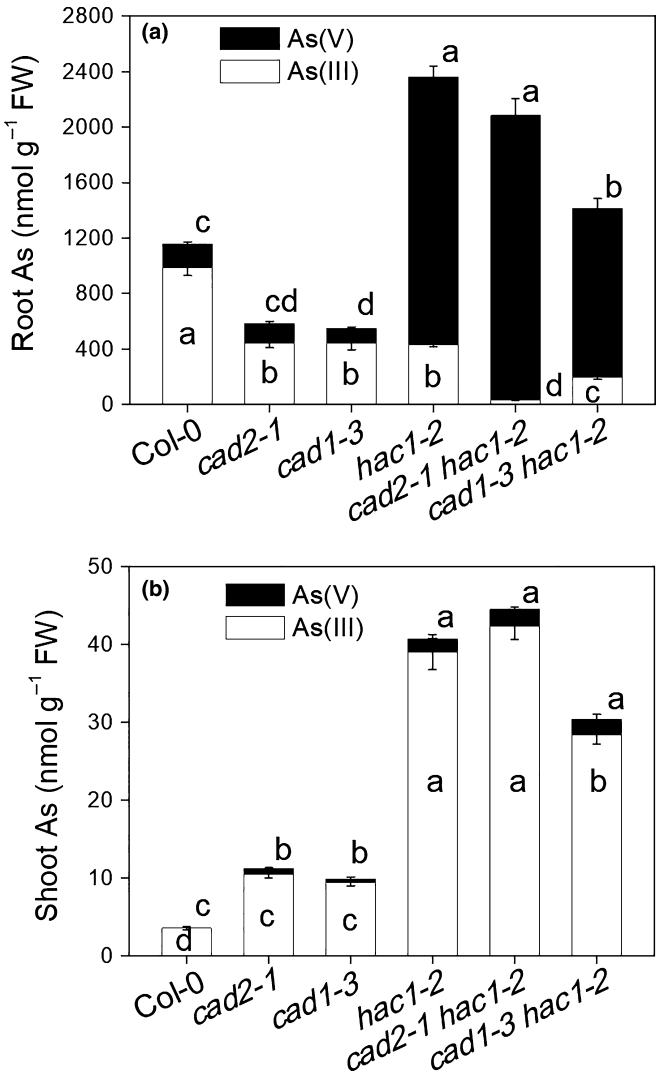

Fig. 7 Arsenic (As) speciation in (a) the roots and (b) shoots of *Arabidopsis thaliana* Col-0, *hac1*, *cad2-1*, *cad1-3* and the double mutants of *hac1 cad2-1* and *hac1 cad1-3* after exposure to 5 μM arsenate (As(V)) (without phosphate) for 1 d. Data are means ± SE ($n = 4$). Different letters above bars represent significant difference at $P < 0.05$.

As(III) efflux to the external medium in the *PvACR3*-expressing lines. A closer examination of the data by Chen *et al.* (2013) revealed that the amount of As(III) extruded into the medium was barely detectable in both Col-0 and transgenic lines, suggesting that their experimental method was not optimized to allow a reliable determination of As(V) uptake and As(III) efflux. Also different from the study of Chen *et al.* (2013) is our observation of a much larger enhancement of root-to-shoot As translocation and shoot As accumulation in the *PvACR3* transgenic lines (Fig. 1). This enhancement occurred regardless of whether As was supplied to plants in the form of As(V) or As(III) (Fig. 1). Although PvACR3 has been shown to be a vacuolar As(III) transporter in the gametophyte of *P. vittata* (Indriolo *et al.*, 2010), PvACR3 was found to be localized to the plasma membrane in both the root and leaf cells of *A. thaliana* plants heterologously expressing *PvACR3:GFP* (Fig. S3) (also Chen *et al.*, 2013). In transgenic *A. thaliana* lines, PvACR3 probably acts as a plasma membrane-localized As(III) efflux transporter for the loading of As(III) into the xylem. The fact that PvACR3 did not enhance As (III) efflux to the external medium could be explained by a preferential accumulation of the protein in the endodermis and the stele in the roots of the transgenic plants (Fig. S4). Although the CaMV 35S promoter used to drive the expression of *PvACR3:GFP* is a constitutive promoter, it is not unusual that such a promoter can lead to a preferential expression in the vascular tissues (Benfey *et al.*, 1989). Thus, decreasing As(III) efflux to the external medium by disrupting *HAC1* and enhancing As(III) efflux to the xylem by expressing *PvACR3* are sufficient to induce As hyperaccumulation in *A. thaliana* shoots. These are the two key traits postulated to explain As hyperaccumulation in *P. vittata* (Su *et al.*, 2008; Zhao *et al.*, 2009). Indeed, As speciation analysis of *P. vittata* roots exposed to As(V) showed relatively low percentages (13–19%) of As(III) (Zhao *et al.*, 2003), suggesting that As(V) reduction is limited in *P. vittata* roots, which is similar to *A. thaliana hac1* mutants. It would be interesting to investigate whether *P. vittata* possesses functional HAC1-like enzymes.

Although HAC1 plays an important role in As(V) reduction in *A. thaliana* roots (Figs 3, 6, 7), there probably exist other As(V) reductases or non-enzymatic As(V) reduction mechanisms that contribute to As(V) reduction in the *hac1* mutants (Chao *et al.*, 2014). As(III) produced by these additional reduction mechanisms is then loaded into the xylem via PvACR3, as well as indigenous As(III) transporters, such as AtNIP3;1 (Xu *et al.*, 2015). The action of PvACR3 may also enhance As(V) reduction in the roots by alleviating the feedback inhibition of As(III), thus explaining the decreased concentrations of not only As(III), but also As(V), in the roots of *hac1 PvACR3* plants (Fig. 3). Different from *hac1 PvACR3* plants, the *hac1 pho1* double mutant had a much lower As concentration in the shoots than the *hac1* single mutant (Fig. 6). The phenotype of the *hac1 pho1* double mutant with a markedly decreased As accumulation in the shoots suggests that As(V) is also loaded into the xylem via PHO1. This mechanism is important in the *hac1* background because of the build-up of As(V) in the roots, but not in the Col-0 background because most of the As(V) taken up was reduced to As(III) (Figs 3, 6). Therefore, xylem loading of As(V) mediated by PHO1 is important for shoot As accumulation in *hac1* plants, whereas As(III) is probably the predominant form of As transported to the shoots in Col-0 and *hac1 PvACR3* plants. In the shoots, however, most of the As was in the form of As(III), even in the *hac1* background, suggesting a strong capacity of As(V) reduction that is little affected by *HAC1* mutation. As(V) reduction in the shoots may be mediated by other As(V) reductases or non-enzymatic reactions (Chao *et al.*, 2014).

Another possible contributing factor in As hyperaccumulation in *P. vittata* is that most of the As in the roots is not complexed with thiol compounds and hence is highly mobile for root-to-shoot translocation (Zhao *et al.*, 2009). The shoot-to-root As concentration ratio was significantly higher in the *cad1-3* and *cad2-1* mutants than in Col-0 (Fig. 7), consistent with a higher As mobility in these mutants. However, this effect is far smaller than that caused by either *HAC1* mutation or *PvACR3* expression (Figs 3, 7). Combining thiol mutants with *HAC1* mutation also

did not increase the root-to-shoot As translocation beyond the level observed in the *hac1* mutant (Fig. 7). These results suggest that the effect on root-to-shoot As translocation ranks in the following order: As(III) xylem loading mediated by PvACR3 > loss of function of *HAC1* > limited As(III)–thiol complexation in the roots.

Comparisons of the contribution of GSH, PCs, HAC1 and PvACR3 to As tolerance

The hypersensitivity of *cad1-3* and *cad2-1* to As(V) or As(III) demonstrates the critical roles of PCs and GSH in As tolerance in *A. thaliana* (Ha *et al.*, 1999; Li *et al.*, 2006; Liu *et al.*, 2010) (also Fig. S8; Table 1). HAC1 is also important for tolerance to As(V), but not to As(III) (Chao *et al.*, 2014; Sanchez-Bermejo *et al.*, 2014). However, the *hac1* mutant showed As(V) sensitivity at a much higher As(V) concentration than either *cad1-3* or *cad2-1*, even though *cad1-3* and *cad2-1* accumulated much lower levels of As in both the roots and shoots than *hac1* (Fig. S8; Table 1). Moreover, *hac1 cad1-3* and *hac1 cad2-1* were no more sensitive to As(V) than *cad1-3* and *cad2-1* single mutants, respectively. These results support the notion that the internal detoxification of As via complexation with thiol compounds represents a fundamental mechanism of As tolerance in As nonhyperaccumulating plants that is required even at relatively low levels of As exposure, whereas HAC1-mediated As(V) reduction and subsequent As (III) efflux confers As(V) tolerance only at high levels of As(V) exposure. Interestingly, *hac1 cad2-1* roots contained As almost exclusively in the form of As(V) after exposure to 5 μM As(V) for 1 d (Fig. 7), demonstrating that the double mutant has lost most of the As(V) reduction capacity. This result suggests that GSH is required as a reductant for either enzymatic or non-enzymatic As (V) reduction.

Unlike As nonhyperaccumulators, only a very small proportion of As in *P. vittata* is complexed with thiol compounds (Webb *et al.*, 2003; Zhao *et al.*, 2003; Zhang *et al.*, 2004; Pickering *et al.*, 2006), suggesting a limited role of PCs in As tolerance in the hyperaccumulator. Vacuolar sequestration of As(III) appears to be the key mechanism of As tolerance in *P. vittata*, with PvACR3 playing an important role in this process (Indriolo *et al.*, 2010). The expression of *PvACR3* in *A. thaliana* significantly increased its tolerance to both As(V) and As(III) (Fig. S2). This result is in agreement with the study by Chen *et al.* (2013). However, their suggestion that PvACR3 enhances As tolerance by extruding As(III) from root cells to the external medium is not supported by our data (Fig. 1). Instead, PvACR3 probably mediates the xylem loading of As(III), thus reducing the build-up of As(III) in the root cells. It has been shown that, in cowpea (*Vigna unguiculata*), root growth is particularly sensitive to As(V) exposure, with As preferentially accumulating in the root apex, causing damage to the meristem (Kopittke *et al.*, 2012). By exporting As(III) from the roots to the less sensitive shoot tissues, PvACR3 may enhance the overall As tolerance of the plant. This hypothesis is supported by the grafting experiment, which shows that *PvACR3* expressed in the root alone is sufficient to explain the enhanced As tolerance in *PvACR3*-expressing *A. thaliana* plants (Fig. 2). *PvACR3* expression in the *hac1* background also partially rescued the As(V)-sensitive phenotype caused by *HAC1* mutation (Fig. S5), which may be attributed to decreased As(III) concentration in the roots (Fig. 3). Despite increased As tolerance in *PvACR3*-expressing *A. thaliana*, the level of tolerance is still below that in *P. vittata*, suggesting the existence of other tolerance mechanisms. Of the genes tested in the present study, their relative importance to As tolerance ranks in the following order: thiol production by PCS1 and γ-ECS > xylem loading of As(III) by PvACR3 > As(V) reduction by HAC1.

Taken together, our study demonstrates that As hyperaccumulation can be engineered in *A. thaliana* by knocking out the *HAC1* gene and expressing *PvACR3*. The same approach may be applied to high-biomass plant species for the purpose of phytoremediation of As-contaminated soil. The expression of *PvACR3* also enhances As tolerance, although not to the level found in *P. vittata*.

Acknowledgements

We thank Professor Lena Ma for the provision of *Pteris vittata* plants. This work was supported by the National Natural Science Foundation of China (31520103914 to F-J.Z. and D.E.S.), the US National Science Foundation (NSF1258097 to J.A.B. and D.E.S.) and the US National Institutes of Health (grants 2R01GM078536 and 2P4ES007373-19A1 to D.E.S.).

Author contributions

F-J.Z., J.A.B. and D.E.S. designed the research. C.W., G.N., E.S.B. and Y.C. performed the experiments and analysed the data. F-J.Z., C.W., J.A.B. and D.E.S. wrote the paper with contributions from all the authors.

References

Ali W, Isner JC, Isayenkov SV, Liu WJ, Zhao FJ, Maathuis FJM. 2012. Heterologous expression of the yeast arsenite efflux system ACR3 improves *Arabidopsis thaliana* tolerance to arsenic stress. *New Phytologist* **194**: 716–723.

Benfey PN, Ren L, Chua NH. 1989. The CaMV S-35 enhancer contains at least 2 domains which can confer different developmental and tissue-specific expression patterns. *EMBO Journal* **8**: 2195–2202.

Chao DY, Chen Y, Chen JG, Shi SL, Chen ZR, Wang CC, Danku JM, Zhao FJ, Salt DE. 2014. Genome-wide association mapping identifies a new arsenate reductase enzyme critical for limiting arsenic accumulation in plants. *PLoS Biology* **12**: e1002009.

Chen YS, Xu WZ, Shen HL, Yan HL, Xu WX, He ZY, Ma M. 2013. Engineering arsenic tolerance and hyperaccumulation in plants for phytoremediation by a *PvACR3* transgenic approach. *Environmental Science & Technology* **47**: 9355–9362.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**: 735–743.

Cobbett CS, May MJ, Howden R, Rolls B. 1998. The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in gamma-glutamylcysteine synthetase. *Plant Journal* **16**: 73–78.

Delhaize E, Randall PJ. 1995. Characterization of a phosphate accumulator mutant of *Arabidopsis thaliana*. *Plant Physiology* **107**: 207–213.

Duan GL, Kamiya T, Ishikawa S, Arao T, Fujiwara T. 2012. Expressing *ScACR3* in rice enhanced arsenite efflux and reduced arsenic accumulation in rice grains. *Plant and Cell Physiology* **53**: 154–163.

González E, Solano R, Rubio V, Leyva A, Paz-Ares J. 2005. PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in *Arabidopsis*. *Plant Cell* **17**: 3500–3512.

Ha SB, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS. 1999. Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell* **11**: 1153–1163.

Howden R, Andersen CR, Goldsbrough PB, Cobbett CS. 1995a. A cadmium-sensitive, glutathione deficient mutant of *Arabidopsis thaliana*. *Plant Physiology* **107**: 1067–1073.

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS. 1995b. Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiology* **107**: 1059–1066.

Indriolo E, Na G, Ellis D, Salt DE, Banks JA. 2010. A vacuolar arsenite transporter necessary for arsenic tolerance in the arsenic hyperaccumulating fern *Pteris vittata* is missing in flowering plants. *Plant Cell* **22**: 2045–2057.

Isayenkov SV, Maathuis FJM. 2008. The *Arabidopsis thaliana* aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. *FEBS Letters* **582**: 1625–1628.

Kamiya T, Tanaka M, Mitani N, Ma JF, Maeshima M, Fujiwara T. 2009. NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of *Arabidopsis thaliana*. *Journal of Biological Chemistry* **284**: 2114–2120.

Kopittke PM, de Jonge MD, Menzies NW, Wang P, Donner E, McKenna BA, Paterson D, Howard DL, Lombi E. 2012. Examination of the distribution of arsenic in hydrated and fresh cowpea roots using two- and three-dimensional techniques. *Plant Physiology* **159**: 1149–1158.

Li YJ, Dankher OP, Carreira L, Smith AP, Meagher RB. 2006. The shoot-specific expression of gamma-glutamylcysteine synthetase directs the long-distance transport of thiol-peptides to roots conferring tolerance to mercury and arsenic. *Plant Physiology* **141**: 288–298.

Lindsay ER, Maathuis FJM. 2016. *Arabidopsis thaliana* NIP7;1 is involved in tissue arsenic distribution and tolerance in response to arsenate. *FEBS Letters* **590**: 779–786.

Liu WJ, Wood BA, Raab A, McGrath SP, Zhao FJ, Feldmann J. 2010. Complexation of arsenite with phytochelatins reduces arsenite efflux and translocation from roots to shoots in Arabidopsis. *Plant Physiology* **152**: 2211–2221.

Ma JF, Yamaji N, Mitani N, Xu XY, Su YH, McGrath SP, Zhao FJ. 2008. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences, USA* **105**: 9931–9935.

Ma LQ, Komar KM, Tu C, Zhang WH, Cai Y, Kennelley ED. 2001. A fern that hyperaccumulates arsenic. *Nature* **409**: 579.

Meharg AA, Williams PN, Adomako E, Lawgali YY, Deacon C, Villada A, Cambell RCJ, Sun G, Zhu YG, Feldmann J *et al.* 2009. Geographical variation in total and inorganic arsenic content of polished (white) rice. *Environmental Science & Technology* **43**: 1612–1617.

Minocha R, Thangavel P, Dhankher OP, Long S. 2008. Separation and quantification of monothiols and phytochelatins from a wide variety of cell cultures and tissues of trees and other plants using high performance liquid chromatography. *Journal of Chromatography A* **1207**: 72–83.

Pickering IJ, Gumaelius L, Harris HH, Prince RC, Hirsch G, Banks JA, Salt DE, George GN. 2006. Localizing the biochemical transformations of arsenate in a hyperaccumulating fern. *Environmental Science & Technology* **40**: 5010–5014.

Pickering IJ, Prince RC, George MJ, Smith RD, George GN, Salt DE. 2000. Reduction and coordination of arsenic in Indian mustard. *Plant Physiology* **122**: 1171–1177.

Quaghebeur M, Rengel Z. 2004. Arsenic uptake, translocation and speciation in *pho1* and *pho2* mutants of *Arabidopsis thaliana*. *Physiologia Plantarum* **120**: 280–286.

Raab A, Williams PN, Meharg A, Feldmann J. 2007. Uptake and translocation of inorganic and methylated arsenic species by plants. *Environmental Chemistry* **4**: 197–203.

Rouached H, Stefanovic A, Secco D, Arpat AB, Gout E, Bligny R, Poirier Y. 2011. Uncoupling phosphate deficiency from its major effects on growth and transcriptome via PHO1 expression in Arabidopsis. *Plant Journal* **65**: 557–570.

Sanchez-Bermejo E, Castrillo G, del Llano B, Navarro C, Zarco-Fernandez S, Jorge Martinez-Herrera D, Leo-del Puerto Y, Munoz R, Camara C, Paz-Ares J *et al.* 2014. Natural variation in arsenate tolerance identifies an arsenate reductase in *Arabidopsis thaliana*. *Nature Communications* **5**: 4617.

Shi S, Wang T, Chen Z, Tang Z, Wu ZC, Salt DE, Chao DY, Zhao FJ. 2016. OsHAC1;1 and OsHAC1;2 function as arsenate reductases and regulate arsenic accumulation. *Plant Physiology* **172**: 1708–1719.

Shin H, Shin HS, Dewbre GR, Harrison MJ. 2004. Phosphate transport in *Arabidopsis*: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. *Plant Journal* **39**: 629–642.

Song W-Y, Park J, Mendoza-Cozatl DG, Suter-Grotemeyer M, Shim D, Hortensteiner S, Geisler M, Weder B, Rea PA, Rentsch D *et al.* 2010. Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. *Proceedings of the National Academy of Sciences, USA* **107**: 21187–21192.

Song W-Y, Yamaki T, Yamaji N, Ko D, Jung K-H, Fujii-Kashino M, An G, Martinoia E, Lee Y, Ma JF. 2014. A rice ABC transporter, OsABCC1, reduces arsenic accumulation in the grain. *Proceedings of the National Academy of Sciences, USA* **111**: 15699–15704.

Su YH, McGrath SP, Zhu YG, Zhao FJ. 2008. Highly efficient xylem transport of arsenite in the arsenic hyperaccumulator *Pteris vittata*. *New Phytologist* **180**: 434–441.

Wang PT, Zhang WW, Mao CZ, Xu GH, Zhao FJ. 2016. The role of OsPT8 in arsenate uptake and varietal difference in arsenate tolerance in rice. *Journal of Experimental Botany* **67**: 6051–6059.

Webb SM, Gaillard JF, Ma LQ, Tu C. 2003. XAS speciation of arsenic in a hyper-accumulating fern. *Environmental Science & Technology* **37**: 754–760.

Xu JM, Shi SL, Wang L, Tang Z, Lv TT, Zhu XL, Ding XM, Wang YF, Zhao FJ, Wu ZC. 2017. OsHAC4 is critical for arsenate tolerance and regulates arsenic accumulation in rice. *New Phytologist* **215**: 1090–1101.

Xu WZ, Dai WT, Yan HL, Li S, Shen HL, Chen YS, Xu H, Sun YY, He ZY, Ma M. 2015. *Arabidopsis* NIP3;1 plays an important role in arsenic uptake and root-to-shoot translocation under arsenite stress conditions. *Molecular Plant* **8**: 722–733.

Xu XY, McGrath SP, Zhao FJ. 2007. Rapid reduction of arsenate in the medium mediated by plant roots. *New Phytologist* **176**: 590–599.

Zhang WH, Cai Y, Downum KR, Ma LQ. 2004. Thiol synthesis and arsenic hyperaccumulation in *Pteris vittata* (Chinese brake fern). *Environmental Pollution* **131**: 337–345.

Zhao FJ, Dunham SJ, McGrath SP. 2002. Arsenic hyperaccumulation by different fern species. *New Phytologist* **156**: 27–31.

Zhao FJ, Ma JF, Meharg AA, McGrath SP. 2009. Arsenic uptake and metabolism in plants. *New Phytologist* **181**: 777–794.

Zhao FJ, McGrath SP, Meharg AA. 2010. Arsenic as a food-chain contaminant: mechanisms of plant uptake and metabolism and mitigation strategies. *Annual Review of Plant Biology* **61**: 535–559.

Zhao FJ, Wang JR, Barker JHA, Schat H, Bleeker PM, McGrath SP. 2003. The role of phytochelatins in arsenic tolerance in the hyperaccumulator *Pteris vittata*. *New Phytologist* **159**: 403–410.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Genotyping of various mutants and expressing lines used in the present study.

Fig. S2 Arsenate and arsenite tolerance of transgenic *Arabidopsis thaliana* expressing *PvACR3*.

Fig. S3 Subcellular localization of PvACR3 in *Arabidopsis thaliana*.

Fig. S4 Preferential expression of PvACR3 in the endodermis and stele of *Arabidopsis thaliana* roots.

Fig. S5 Effect of combining *PvACR3* expression with *HAC1* mutation on arsenate tolerance in *Arabidopsis thaliana*.

Fig. S6 Expression levels of *PvACR3* and *HAC1* genes in *Arabidopsis thaliana hac1* mutant expressing *PvACR3*, and the growth phenotypes of the different lines grown in a potting medium with or without 10 mg kg^{-1} As(V).

Fig. S7 The concentrations of nonprotein thiols in the roots and shoots of Col-0, *cad2-1*, *cad1-3*, *hac1-2*, *cad2-1 hac1-2* and *cad1-3 hac1-2* of *Arabidopsis thaliana*.

Fig. S8 Arsenate tolerance in *hac1*, *cad2-1*, *cad1;3* and double mutants of *cad2-1 hac1* and *cad1;3 hac1*.

Table S1 Primers used for the genotyping of different lines of *Arabidopsis thaliana*

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