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

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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*), PC synthase (*PCS1*) and phosphate effluxer (*PHO1*), and 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 localised 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 *HAC1* gene and expressing *PvACR3*. PvACR3 and HAC1 also affect As tolerance, but not to the extent of γ-ECS and PCS1.

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

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 characterised 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 an approximately 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-weeks growth in a potting mix spiked with 7.5 mg kg^{-1} As(V) (Chao *et al.*, 2014). In rice, *oshac1;1 oshac1;2* double mutant and *oshac4* single mutant had 2.3 and 3 fold, respectively, higher As concentrations in the shoots compared with wild-type (WT) (Shi *et al.*, 2016; Xu *et*

al., 2017). There are several possible reasons for the observed effect of the HACs on the root to shoot As translocation. First, HACs reduces 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 the *HACs* 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 the As(III) to be complexed by thiol compounds and subsequently sequestered in the vacuoles in the roots (Song *et al.*, 2010; Song *et al.*, 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 wild-type plants (Liu *et al.*, 2010). In the As hyperaccumulator *P. 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 the 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 toward the stele for xylem loading (Ma *et al.*, 2008). A high expression of Lsi2 and its polar localisation on the proximal side of the 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 localised 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

Plant materials used included *A. thaliana* wild-type Columbia-0 (Col-0) and *cad1-3*

(PC-deficient mutant), and *cad2-1* (GSH-deficient mutant) (Howden *et al.*, 1995a; Howden *et al.*, 1995b), two T-DNA insertion knockout mutants of *HAC1* (GABI_868F11, SM_3_38332 for *hac1-1* and *hac1-2*, respectively) (Chao *et al.*, 2014), and *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 respective single mutants. Homozygous double mutants were identified by PCR genotyping of the F_2 progeny (Supplementary Fig. S1, Table S1).

To generate the *35S::PvACR3* construct, *PvACR3* (UniProt #FJ751631) was cloned into pCC0869, a pBI121-derived plant transformation vector containing 35S CaMV promoter. The *PvACR3* gene was PCR amplified with the 5' primer 5'-GCTCTAGAATGGAGAACTCAAGCG-3' (XbaI) and the 3' primer 5'- TCCCCC GGGCTAAACAGAAGGCCCCTTC-3' (SmaI) using cDNA derived from arsenate-grown gametophytes of *P. vittata*, and the resulting PCR fragment confirmed by sequencing. The resulting fragment was inserted into XbaI and SmaI-linearized pCC0869 vector. *A. 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 T3 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 F2 progeny by PCR genotyping. Second, *PvACR3* was expressed in *hac1* directly. *PvACR3* was cloned into the *2X*35S promoter cassette of pMDC32 between AscI and PacI restriction sites by recombination of the following primers: 5'-CGGGCCCCCCCTCGAGGCGCGCCATGGAGAACTCAAGCGCGGAGC-3' (AscI) and 5'-CCGCTCTAGAACTAGTTAATTAACTAAACAGAAGGCCCCTTCC-3' (PacI), using the ClonExpress™ II one step cloning kit (Vazyme). The binary vector pMDC32-*PvACR3* was transformed into *Agrobacterium* strain GV3101 by

freeze-thaw method. The *Agrobacterium* culture was used to transform *A. thaliana hac1-2* by *Agrobacterium*-mediated dip floral transformation (Clough & Bent, 1998). Homozygous lines were identified in the T3 generation, and three lines with relatively high levels of *PvACR3* expression in the roots were selected.

Arsenic tolerance assays

A. thaliana seeds were surface-sterilized and sown on agar plates containing MS medium (1/2 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 5 plates. All plates were placed at 4°C in the dark for 2 days to synchronize germination. The plates were then placed vertically in a growth chamber at 22°C with a 16-h light/8-h dark regime. Root length and shoot fresh weight were determined after 2-week 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 4 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 analysis of As speciation and concentration.

PvACR3 hac1 and WT seeds were germinated on 1/2 MS medium without As for 1 week. Seedlings were transplanted into an As contaminated soil for 3 weeks. The soil contained 101 mg kg^{-1} total As due to nearby mining activities. Each pot was filled with 0.3 kg soil and planted with either 4 *A. thaliana* plants or 1 plant of *P. vitatta* (2-3 frond stage), with 4 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 5 replicates per line. Plants were grown in a growth chamber at 22°C with a 16-h light/8-h dark regime. *A. thaliana* plants were harvested 3 weeks after transplanting, whilst *P. vittata* plants were harvested 3 and 6 weeks after transplanting.

RNA extraction and Semi-quantitative RT-PCR

Total RNAs were extracted from 10-day-old *A. thaliana* roots and shoots using the RNeasy plant Mini Kit (Biotech). Reverse transcription was carried out using the R233-01 kit (Vazyme). Semi-quantitative RT-PCR was done in a reaction mixture of 20 µl of 2 X Taq Master Mix (Vazyme) for 30 cycles. *Actin2* was used as the reference gene (primer, forward 5'-TCACAGCACTTGCACCAAGCA-3', reverse 5'-AACGATTCCTGGACCTGCCTCA-3'). *HAC1* and *PvACR3* were PCR amplified using the primer sets 5'-GAAGATGTTGAGACCGTTGATGTTT-3' (forward) and 5'-TCACTTTCAAGTTTCAAGTGCCGAT-3' (reverse) for *HAC1* and 5'-ATGGAGAACTCAAGCGCG-3' (forward) and 5'-GACCCCACCCAGCATTTCAT -3' (reverse) for *PvACR3*.

Subcellular localisation of PvACR3

To investigate the subcellular localization of PvACR3 expressed in *A. thaliana*, a *35S::PvACR3-GFP* fusion construct was generated. The *Aequorea coerulescens* GFP (*AcGFP*) coding region was amplified from plasmid pUC-AcGFP and inserted into the SmaI and EcoRI 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'-GCTCTAGAATGGAGAACTCAAGCG-3'(XbaI, forword) and 5'- TCCCCCGGGAACAGAAGGCCCCTTCCTC-3' (SmaI, reverse) then cloned in frame to *AcGFP*. The final construct was confirmed by sequencing. The construct was introduced to *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., Germany). To label *A. thaliana* seedlings with the plasma membrane dye FM4-64 (T13320, Invitrogen), 5-day-old whole seedlings grown on petri dishes were incubated with 1 μM FM4-64 in water for 15 min. Seedlings were rinsed in distilled water and imaged immediately. To further confirm the subcellular localisation of PvACR3, total microsomal membrane fractions were isolated from 4-week-old whole transgenic *A. thaliana* expressing *PvACR3*. Aqueous two-phase extractions performed as previously described (Indriolo *et al.*, 2010).

Determination of As speciation and total As concentration

At the end of As exposure, roots were desorbed of the 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 (～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), 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/$HClO_4$ (vol:vol = 85:15) 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 non-protein thiols

After 24 h exposure to As(V), *A. thaliana* roots and shoots were separated and immediately frozen in liquid nitrogen. Non-protein thiol compounds (Cys, GSH, PC_2, PC_3 and PC_4) were extracted and quantified using a 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 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 (Supplementary Fig. S2). In the absence of As(III), no significant differences in root growth or shoot fresh weight between WT and transgenic lines were observed (Fig. S2c, d). In the presence of toxic As(III) concentrations (25 - 100 μM As(III)), both lines grew significant better than WT plants (Fig. S2b, c, 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 2 – 3 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

– 5.8 times of those for WT based on root growth, and 2.1 – 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 days. 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 – 34 fold higher As concentrations of As in the shoots (Fig. 1a, b). The ratio of 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*. Expression of *PvACR3* decreased the As(III)% in the roots to 88 – 89%, but increased the 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 day of As(V) exposure (Fig. 1e).

To determine whether the expression of *PvACR3* in the root, the shoot or both is important in conferring As tolerance in *A. thaliana*, four types of reciprocal grafts between WT (Col-0) 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) while self-grafted *PvACR3*-E8 plants did not. When WT shoot scions were grafted to *PvACR3*-E8 rootstocks, the resulting plants showed As tolerance comparable to 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 localised to the plasma membrane in transgenic *A. thaliana* plants

The subcellular localisation of the PvACR3 protein in *A. thaliana* was determined by expressing *PvACR3:AcGFP* under the control of 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-localise with the red fluorescence of FM4-64 in both leaves and roots (Supplementary Fig. S3a), indicating that the PvACR3:AcGFP fusion protein localises to the plasma membrane. Furthermore, *PvACR3:AcGFP* appears to be preferentially expressed in the endodermis and the stele of the transgenic *A. thaliana* roots (Supplementary Fig. S4).

The plasma membrane localisation of PvACR3:AcGFP was also confirmed by protein immunoblotting using antibodies to GFP after membrane purification using two-phase extraction (Supplementary 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 localisation of the ACR3:AcGFP fusion protein.

354 Combining *HAC1* mutation with *PvACR3* expression leads to As
355 hyperaccumulation in *A. thaliana*
356
357 Because mutation of *HAC1* also results in a large increase in As accumulation in the
358 shoots (Chao *et al.*, 2014), we tested the combined effect of *HAC1* mutation with
359 *PvACR3* expression. We first crossed *PvACR3*-E8 with two T-DNA insertion *HAC1*
360 knockout lines and obtained homozygous lines combining *PvACR3* expression with
361 *HAC1* mutation. The As(V) tolerance results of *PvACR3*-E8 *hac1-1* and
362 *PvACR3*-E8 *hac1-2* are similar, so only the dataset of the former is shown here.
363 Consistent with the results shown in Fig. S2 and those reported by Chao *et al.*
364 (2014), *PvACR3* expression increased As(V) tolerance in *A. thaliana*, whereas *hac1*
365 mutants were more sensitive to As(V) than WT plants (Supplementary Fig. S5,
366 Table 1). In this experiment, the highest As(V) concentration (250 μM) did not
367 significantly inhibit root or shoot growth of *PvACR3*-E8; therefore the exact EC_{50}
368 could not be estimated (Table 1). Expression of *PvACR3* in *hac1* mutants enhanced
369 the As(V) tolerance to a level that was similar to or higher than that of WT plants,
370 but lower than that of *PvACR3*-E8 plants, especially at the high (250 μM) As(V)
371 concentration (Fig. S5, Table 1).
372 We then determined As accumulation and speciation in hydroponically grown
373 plants exposed to 5 μM As(V) for 1 day. *HAC1* mutation resulted in a 35% decrease
374 in the root As(III) concentration, but a 16 – 24 fold increase in the root As(V)
375 concentration, compared with WT (Fig. 3a). The percentage of As(III) in the root
376 (relative to total As) decreased from 90% in WT plants to 19 – 27% in *hac1* mutants,
377 which is indicative of a loss of function of a key As(V) reductase. *PvACR3*
378 expression in the Col-0 background decreased As(III) concentration in the roots by
379 74%, but had little effect on the As(V) concentration. Combining *PvACR3*
380 expression with *HAC1* mutation decreased both As(III) and As(V) concentrations in
381 the roots compared with the *hac1* mutants. *HAC1* mutation, *PvACR3* expression and
382 the combination of the two genetic events produced striking phenotypes in As
383 accumulation *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 – 117 fold (Fig. 3b). The increase in the shoot As concentration in *PvACR3*-E8 *hac1* plants was more 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 approximately 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 (Supplementary Fig. S6a). The three transgenic lines, *hac1-2* and Col-0 were grown for 3 weeks 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 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). Expression of *PvACR3* in *hac1-2* enhanced As accumulation in the shoots much further, 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. Shoot biomass after 3-week growth was not significantly different between different *A. thaliana* lines (Fig. 5a). Compared with Col-0, 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 weeks, there was no significant increase in the frond biomass (Fig. 5c). There was also no significant increase in As concentration in the fronds 3 weeks after transplanting compared with the initial As concentration at the time of transplanting (Fig. 5d). Six weeks after transplanting, As concentration in *P. vittata* fronds increased from the initial level of 14 mg kg^{-1} to 54 mg kg^{-1} (Fig. 5d). The net increase in frond As concentration was comparable to the shoot As concentrations of the *PvACR3 hac1-2* lines grown in the same soil over 3 weeks.

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 *pho1-2* mutant (Delhaize & Randall, 1995) with *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 approximately 70% and 40% inhibition of the shoot and root biomass, respectively (Fig. 6a). This phenotype is similar to that reported before (Delhaize & Randall, 1995; Rouached *et al.*, 2011). The *hac1-1* mutant was also smaller than WT (by approximately 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 roots and shoots was determined after plants were exposed to 5 μM As(V) for 1 day 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 had 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

444 the form of As(V), accounting for 58% of the total As, whereas most of the
445 increased shoot As concentration was in the form of As(III). The As accumulation
446 phenotype in *hac1-1* was reduced by 85% in the *hac1-1 pho1-2* double mutant,
447 although total As concentrations in the roots and shoots of the double mutant were
448 still significantly higher than those of Col-0 (by 20% and 6 fold, receptively) (Fig.
449 6b, c). The As(III)% in the roots of the double mutant was 59%, which was lower
450 than Col-0 and *pho1-2* (95 – 96%) but higher than *hac1-2* (42%). The results
451 suggest that *PHO1* mutation had no impact on As accumulation in the Col-0
452 background, but greatly suppressed As translocation from the roots to the shoots in
453 the *hac1-2* background.
454
455 Effects of GSH and PC mutants in combination with HAC1 mutation on As
456 tolerance and accumulation
457
458 It is well known that GSH and PCs are crucial for As detoxification through the
459 formation of As(III)-thiol complexes (Ha *et al.*, 1999; Pickering *et al.*, 2000; Liu *et*
460 *al.*, 2010). Moreover, the sequestration of the As(III)-thiol complexes in the root
461 vacuoles reduces the root to shoot translocation of As in *A. thaliana* Col-0 (Liu *et*
462 *al.*, 2010). Here, we investigated the effects of combining mutations that reduce
463 GSH (*cad2-1*) or PC (*cad1-3*) concentrations (Howden *et al.*, 1995a; Howden *et al.*,
464 1995b; Cobbett *et al.*, 1998) with *hac1-1* and *hac1-2* mutants (Chao *et al.*, 2014) on
465 As tolerance and accumulation. As the double mutants of *cad2-1 hac1-1* and *cad1-3*
466 *hac1-1* behaved similarly to *cad2-1 hac1-2* and *cad1-3 hac1-2*, respectively, only
467 one set of data are presented. In addition to genotyping based on the mutated genes,
468 we also determined the concentrations of Cys, GSH and PCs in the roots and shoots
469 of WT, single and double mutants after exposure to 5 μM As(V) for 1 day. The
470 results are consistent with expectations, with *cad2-1* and *cad2-1 hac1-2* containing
471 lower levels of GSH and PCs than WT and *cad1-3* and *cad1-3 hac1-2* containing
472 almost no PCs (Supplementary Fig. S7).
473 Tolerance to As(V) was assessed by measuring root and shoot biomass after

plants were 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 10 times lower than WT based on root growth and approximately 20 times lower than WT based on shoot growth (Supplementary Fig. 8, 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 mutant, respectively, with similar EC_{50} values between the single and double mutants (Table 1).

After exposure to 5 μM As(V) for 1 day, *hac1-2* accumulated 2 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 (~50%) but more As in the shoots (~3 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* had 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 had lower 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 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 *HAC1* mutation (Chao *et al.*, 2014) or expression

of *PvACR3* in the Col-0 background of *A. thaliana* (Chen *et al.*, 2013) markedly increases As accumulation in the above-ground 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 additive of the two events alone. Moreover, when *hac1 PvACR3* plants were grown in an As contaminated soil for 3 weeks, they accumulated As in the shoots to levels comparable to the As hyperaccumulating fern *P. vittata* grown on the same soil for 6 weeks after transplanting (Fig. 5).

Consistent with the 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 and 3). This result is different from the study of Chen *et al.* (2013), which reported increased As(III) efflux to the external medium in the *PvACR3* expressing lines. A closer examination of the data by Chen *et al.* (2013) reveals 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 was shown to be a vacuolar As(III) transporter in the gametophyte of *P. vittata* (Indriolo *et al.*, 2010), PvACR3 was found to be localised to the plasma membrane in both the root and leaf cells of *A. thaliana* plants heterologously expressing *PvACR3:GFP* (Supplementary Fig. S3) (also Chen *et al.*, 2013). In transgenic *A. thaliana* lines, PvACR3 likely acts as a plasma membrane localised

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 (Supplementary Fig. S4). Although the CaMV35S 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). In fact, 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.

Despite HAC1 playing an important role in As(V) reduction in *A. thaliana* roots (Figs. 3, 6, 7), there likely 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 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, *hac1 pho1* double mutant had a much lower As concentration in the shoots than *hac1* single mutant (Fig. 6). The phenotype of *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 and 6). Therefore, xylem loading

564 of As(V) mediated by PHO1 is important for shoot As accumulation in *hac1* plants,
565 whereas As(III) is likely to be the predominant form of As transported to the shoots
566 in Col-0 and *hac1 PvACR3* plants. In the shoots, however, most of the As was in the
567 form of As(III) even in the *hac1* background, suggesting a strong capacity of As(V)
568 reduction that is little unaffected by *HAC1* mutation. As(V) reduction in the shoots
569 may be mediated by other As(V) reductases or non-enzymatic reactions (Chao *et al.*,
570 2014).

571 Another possible contributing factor in As hyperaccumulation in *P. vittata* is
572 that most of the As in the roots is not complexed with thiol compounds and hence is
573 highly mobile for root to shoot translocation (Zhao *et al.*, 2009). The shoot to root
574 As concentration ratio was significantly higher in the *cad1-3* and *cad2-1* mutants
575 than in Col-0 (Fig. 7), consistent with a higher As mobility in these mutants.
576 However, this effect is far smaller than that caused by either *HAC1* mutation or
577 *PvACR3* expression (Figs. 3 and 7). Combining thiol mutants with *HAC1* mutation
578 also did not increase the root to shoot As translocation beyond the level observed in
579 the *hac1* mutant (Fig. 7). These results suggest that the effect on root to shoot As
580 translocation ranks in the following order: As(III) xylem loading mediated by
581 PvACR3 > loss of function of *HAC1* > limited As(III)-thiol complexation in roots.
582
583 Comparisons of the contribution of GSH, PCs, HAC1 and PvACR3 to As tolerance
584
585 The hypersensitivity of *cad1-3* and *cad2-1* to As(V) or As(III) demonstrates the
586 critical roles of PCs and GSH in As tolerance in *A. thaliana* (Ha *et al.*, 1999; Li *et*
587 *al.*, 2006; Liu *et al.*, 2010) (also Fig. S8, Table 1). HAC1 is also important for the
588 tolerance to As(V), but not to As(III) (Chao *et al.*, 2014; Sanchez-Bermejo *et al.*,
589 2014). However, the *hac1* mutant showed As(V) sensitivity at a much higher As(V)
590 concentration than either *cad1-3* or *cad2-1*, even though *cad1-3* and *cad2-1*
591 accumulated much lower levels of As in both roots and shoots than *hac1* (Fig. S8,
592 Table 1). Moreover, *hac1 cad1-3* and *hac1 cad2-1* were no more sensitive to As(V)
593 than *cad1-3* and *cad2-1* single mutant, respectively. These results support the notion

that 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 day (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 non-hyperaccumulators, only a very small proportions 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). 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 likely mediates 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 has demonstrated that As hyperaccumulation can be engineered in *A. thaliana* by knocking out *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. Expression of *PvACR3* also enhances As tolerance, though not to the level found in *P. vittata*.

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Author contributions

F-J.Z., J.A.B. and D.E.S. designed the research. C.W., G.N.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.

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

Lines	As(V) EC_{50} (μM)		As(III) EC_{50} (μM)	
	Root length	Shoot biomass	Root length	Shoot biomass
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
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		
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		
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] Experiment 1 and Experiments 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 a significant inhibition, EC_{50} could not be estimated and was considered to be higher than the largest As concentration in the experiment.

List of Figures:

Fig. 1 Arsenic accumulation in transgenic *A. thaliana* expressing *PvACR3*. (a, b) Total As concentration in roots (a) and shoots (b) of Col-0 and *PvACR3* expressing lines after plants were exposed to 5 μM As(III) for 2 days. (c, d) The concentrations of As(III) and As(V) in roots (c) and shoots (d) of Col-0 and *PvACR3* expressing lines after plants were exposed to 5 μM As(V) for 2 days in the absence of phosphate. (e) As(V) uptake and As(III) efflux to the external solution after plants were exposed to 5 μM As(V) for 1 day in the absence of phosphate. Data are means ± SE (n = 4). The % values in (e) represents As(III) efflux as a % of As(V) uptake. Different letters above bars indicate significant differences at $P<0.05$.

Fig. 2 Arsenic tolerance of reciprocally grafted *A. thaliana* plants expressing *PvACR3*. Reciprocally grafted seedlings were grown on plates in the absence of arsenic (a) or in the presence of 25 μM arsenite (b); 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). Root growth (c) and shoot fresh weight (d) in the presence of 25 μM arsenite relative to control treatment. Data point are mean ± SE (n = 4). Same letter within graphs represents lines that are not significantly different ($P>0.05$). *PvACR3*-E8 lines was used as a *PvACR3* expressing transgenic line.

Fig. 3 Effect of combining *PvACR3* expression with *HAC1* mutation on As accumulation and speciation in *A. 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 As(V) for 1 day without phosphate. (c) As(V) uptake and As(III) efflux into the nutrient solution after 1-day exposure to 5 μM As(V) without phosphate. Data are means ± SE (n = 4). The % values in (c) represents As(III) efflux as a % of As(V)

uptake. Different letters above bars indicate significant differences at $P<0.05$.

Fig. 4 Arsenic accumulation in transgenic *A. thaliana hac1* mutant expressing PvACR3 grown in a potting medium. (a) Shoot dry weights of different lines as affected by the addition of 10 mg kg^{-1} 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 means ± SE (n=4). The values inside open bars represent the percentage values relative to the control. Different letters above bars represent significant difference at $P < 0.05$.

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

Fig. 6 Effect of combining *PHO1* and *HAC1* mutation on As accumulation in *A. thaliana.* (a) Shoot and root fresh weights 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 day without phosphate. Data are means ± SE (n = 4). Different letters above bars indicate significant differences at $P<0.05$.

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

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858 **Supporting Information**

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

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

861 **Fig. S3** Subcellular localisation of PvACR3 in *A. thaliana*.

862 **Fig. S4** Preferential expression of PvACR3 in the endodermis and stele of *A. thaliana*
863 roots.

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

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

869 **Fig. S7** The concentrations of non-protein thiols in the roots and shoots of Col-0,
870 *cad2-1*, *cad1-3*, *hac1-2*, *cad2-1hac1-2* and *cad1-3 hac1-2* of *A. thaliana*.

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

873 **Table S1** Primers used for genotyping of different lines of *A. thaliana*.

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