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Heterologous Expression of *Pteris vittata* Arsenite Antiporter PvACR3;1 Reduces Arsenic Accumulation in Plant Shoots

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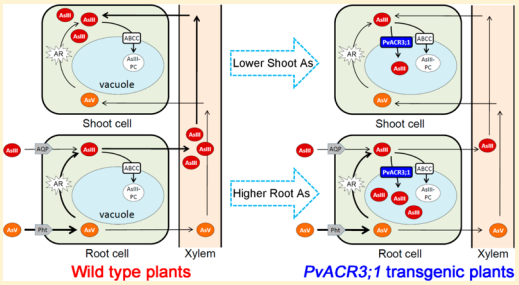
Supporting Information

ABSTRACT: Arsenic (As) is a toxic carcinogen so it is crucial to decrease As accumulation in crops to reduce its risk to human health. Arsenite (AsIII) antiporter ACR3 protein is critical for As metabolism in organisms, but it is lost in flowering plants. Here, a novel *ACR3* gene from As-hyperaccumulator *Pteris vittata*, *PvACR3;1*, was cloned and expressed in *Saccharomyces cerevisiae* (yeast), *Arabidopsis thaliana* (model plant), and *Nicotiana tabacum* (tobacco). Yeast experiments showed that PvACR3;1 functioned as an AsIII-antiporter to mediate AsIII efflux to an external medium. At 5 μM AsIII, *PvACR3;1* transgenic *Arabidopsis* accumulated 14−29% higher As in the roots and 55−61% lower As in the shoots compared to WT control, showing lower As translocation. Besides, transgenic tobacco under 5 μM AsIII or AsV also showed similar results, indicating that expressing *PvACR3;1* gene increased As retention in plant roots. Moreover, observation of PvACR3;1−green fluorescent protein fusions in transgenic *Arabidopsis* showed that PvACR3;1 protein localized to the vacuolar membrane, indicating that PvACR3;1 mediated AsIII sequestration into vacuoles, consistent with increased root As. In addition, soil experiments showed ∼22% lower As in the shoots of transgenic tobacco than control. Thus, our study provides a potential strategy to limit As accumulation in plant shoots, representing the first report to decrease As translocation by sequestrating AsIII into vacuoles, shedding light on engineering low-As crops to improve food safety.



INTRODUCTION

Arsenic (As) is a toxic and carcinogenic metalloid, ranking as the No. 1 contaminant by the Agency for Toxic Substances & Disease Registry. Arsenic is present in the environment as both organic and inorganic forms, with inorganic As being more toxic. In humans, chronic ingestion of As is associated with skin and lung cancers.[1] There are various sources of As, with food crops grown in As-contaminated soils being an important source. For example, in China, daily inorganic As intake is ∼42 μg, with rice being the largest contributor, accounting for ∼60% of total inorganic As intake.[2] Therefore, it is of significance to limit As into the food chain from soil to reduce potential As risks to humans.

Arsenate (AsV) is the dominant As species in aerobic soils[3,4] and is taken up by phosphate transporters in plants.[5] After being taken up, AsV can be rapidly reduced to arsenite (AsIII) in the roots by arsenate reductase.[5−12] Besides AsV reduction, plants have also developed other strategies to cope with As to survive in As-contaminated soils. These include AsIII chelation by sulfhydryl (−SH) groups of peptide phytochelatins (PCs) and subsequent sequestration into intracellular compartments,[13−15] and AsIII efflux from root cells to the external environment.[16−18] In most plants, most As is accumulated in the roots, but some is translocated to plant shoots via the xylem, leading to As accumulation in edible parts including leaves, seeds, and grains.[19] Thus, to reduce As in the food chain, it is essential to decrease As translocation to limit As accumulation in plant shoots.

In plants, sequestration of heavy metals into the root vacuoles can function as a mechanism to reduce their translocation. For example, heavy metal ATPase 3 (OsHMA3) in rice, mediates Cd transport into root vacuoles, reducing Cd in the roots to be loaded into xylem for root-to-shoot Cd translocation.[20,21] AsIII has a high affinity for PCs to form AsIII-PC, which can be sequestrated into vacuoles as a step of As detoxification in cells, thereby affecting As distribution in plant tissues. C-type ATP binding cassette

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transporters (ABCC) in plants mediate vacuolar AsIII-PC sequestration.[13,14] In *Arabidopsis*, AtABCC1 and AtABCC2 are the major AsIII-PC transporters on the vacuolar membrane, so they are essential for As tolerance.[13] In rice, OsABCC1 protein is responsible for AsIII-PC sequestration and knockout of *OsABCC1* gene increases As sensitivity and also As allocation to rice grain.[14] These ABCC transporters affect As translocation in plants, and thus can be used to engineer plants for low As accumulation in edible parts.

Besides ABCC1 transporters, whether other transporters mediate As sequestration into vacuoles in plants has not been fully elucidated. In yeast, AsIII transporter ACR3 (Arsenic Compounds Resistance 3) is localized to the plasma membrane to export AsIII out of the cell.[22] Interestingly, its homologues exist in plants including moss, lycophytes, ferns, and gymnosperms, but not angiosperms.[23] In As-hyperaccumulator *Pteris vittata*,[24] two *ACR3* homologues, *PvACR3* and *PvACR3;1*, were reported, with PvACR3 being localized to the vacuolar membrane and likely effluxing AsIII into the vacuole for sequestration.[23] However, in transgenic *Arabidopsis*, PvACR3 localizes to the plasma membrane and its heterologous expression increases AsIII efflux.[16] Although *PvACR3;1* was reported by Indriolo et al.,[23] it was not investigated in their study so its function is unclear.

Here, in this study, we successfully cloned the *PvACR3;1* full length coding sequence (CDS) from *P. vittata* and tested its function following expression in transgenic yeast and in transgenic plants. We found that *PvACR3;1* expression enhanced As tolerance in yeast by mediating AsIII efflux from cytoplasm into external medium. We also found that *PvACR3;1* expression in *Arabidopsis* and tobacco increased As retention in the roots, thereby decreasing As accumulation in the shoots. Further analysis of its subcellular localization showed that PvACR3;1 was localized to vacuolar membrane, indicating that PvACR3;1 probably mediated As sequestration into vacuoles to increase As retention in the roots, therefore reducing As translocation to the shoots. Thus, our work provides a potential new engineering strategy to decrease As accumulation in plant shoots, reducing As risks to humans.

MATERIALS AND METHODS

Synthesis of *PvACR3;1* cDNA from *Pteris vittata*. Total RNA was extracted from the fronds of As-hyperaccumulator *P. vittata* from Florida, U.S.A. The first-strand cDNA was synthesized from 2 μL total RNA using the EasyScript First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech, China). Then the *PvACR3;1* CDS was amplified by PCR using PrimeSTAR HS DNA Polymerase (Takara Biomedical Technology, Beijing) and the following primers: 5′-ATG GCC GAC AGC ACT CGT TAT GAT C-3′ and 5′-CTA GTA GGG TTC TGC AGG CCA CTT C-3′.

Yeast Vector Construction, Transformation, and Growth Assays. Adapters were added to *PvACR3;1* CDS using the following primers: 5′-gaa aaa acc ccg gat tct aga ATG GCC GAC AGC ACT CGT TAT GAT C-3′ and 5′-taa cta att aca tga ctc gag CTA GTA GGG TTC TGC AGG CCA CTT C-3′ (underlining indicates recombination sequences). The PCR product was then cloned into the GAL1 promoter cassette of pAG413GAL-ccdB (Addgene, http://www.addgene.org/) between *Xba*I and *Xho*I restriction sites by recombination, using the CloneEZ PCR Cloning Kit (Genscript Company), with the constructed binary vector being named pAG413GAL-*PvACR3;1*.

Yeast strain used for heterologous expression of *PvACR3;1* was the Δ*acr3* mutant with BY4741 (MATa *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0) background.[25] High efficiency transformation of yeast described by Gietz et al.[26] was followed. Yeast cells were grown at 30 °C in synthetic defined (SD) medium (0.67% yeast nitrogen base) without amino acids, containing 2% (w/v) glucose or 2% (w/v) galactose (induction medium), supplemented with yeast synthetic dropout without histidine at pH 5.8. For As tolerance assays, yeast was grown in liquid SD medium (with 2% [w/v] glucose) to an OD_{600} of ~1.0 and then subjected to centrifugation and dilution with sterile water. The drop assays were performed on SD plates (with 2% [w/v] galactose) containing 100 or 200 μM AsIII, and 500 or 1000 μM AsV for Δ*acr3* expressing *PvACR3;1*. For As toxicity growth curve assay, yeast was grown in liquid SD medium (with 2% [w/v] glucose) to an OD_{600} of ~1.0 and then subjected to centrifugation. Then the yeast was diluted with 20 mL liquid SD medium (with 2% [w/v] galactose) to an OD_{600} of 0.1 in 100 ml triangular flasks containing 200 μM AsIII or 400 μM AsV. The triangular flasks were incubated on a rotary shaker at 200 rpm at 30 °C and the OD_{600} values of the medium were measured after 6, 12, 18 and 24 h.

Plant Expression Vector Construction and Transgenic Plant Generation. Adapters were added to *PvACR3;1* CDS using the following primers: 5′-acg ggg gac tct aga gga tcc ATG GCC GAC AGC ACT CGT TAT GAT C-3′ and 5′-ggg aaa ttc gag ctc ggt acc CTA GTA GGG TTC TGC AGG CCA CTT C-3′ (underlining indicates recombination sequences). The PCR product was then cloned into the CaMV 35S promoter cassette of pSN1301 (pCAMBIA1301, CAMBIA)[16] between *Bam*HI and *Kpn*I restriction sites by recombination, using the CloneEZ PCR Cloning Kit (Genscript Company), with the constructed binary vector being named pSN1301-*PvACR3;1*. *Agrobacterium* strain C58 was transformed with the binary vector pSN1301-*PvACR3* by electroporation. The Agrobacterium culture was used to transform *Arabidopsis thaliana* Col-0 by *Agrobacterium*-mediated dip floral transformation.[27] Transformation of tobacco leaf explants was carried out following Curtis et al. and Gallois and Marinho.[28,29] Transgenic plants were obtained by hygromycin selection, and further verified by GUS staining and PCR analysis. Homozygous lines were identified in the T3 generation via segregation analysis.

Plant Growth and As Determination. For analysis of As accumulation in *Arabidopsis* seedlings, seeds were surface-sterilized and sown on media containing 1/2 Murashige and Skoog (MS) salts, 1% sucrose and 0.5 g/L MES at pH 5.9, and solidified with 0.8% agar. After 2 d to synchronize germination at 4 °C in the dark, the plates were placed in a growth chamber at 22 °C with a 16-h light/8-h dark regime to facilitate germination. Seedlings, 7 d after germination, were transferred onto plates containing the 1/2 strength MS agar media supplemented with 0, 5, and 10 μM AsIII ($NaAsO_2$). The plants were grown vertically at 22 °C with a 16-h light/8-h dark regime for 7 d, after which root elongations of the plants were measured. Besides, plants under 5 μM AsIII treatments for 7 d were also collected for biomass analysis and As determination.

For analysis of As accumulation in tobacco plants (*N. tabacum*), seeds were germinated and cultivated in a soil for 4 weeks. Uniform 4-wk-old tobacco seedlings were transferred into hydroponic medium, 500 mL of 0.2 strength Hoagland-Arnon nutrient solution (HNS) containing 5 μM AsIII or 5 μM AsV ($Na_2HAsO_4{\cdot}7H_2O$) (3 plants for each vessel) and cultivated for 1 d. Then the plant roots were washed with

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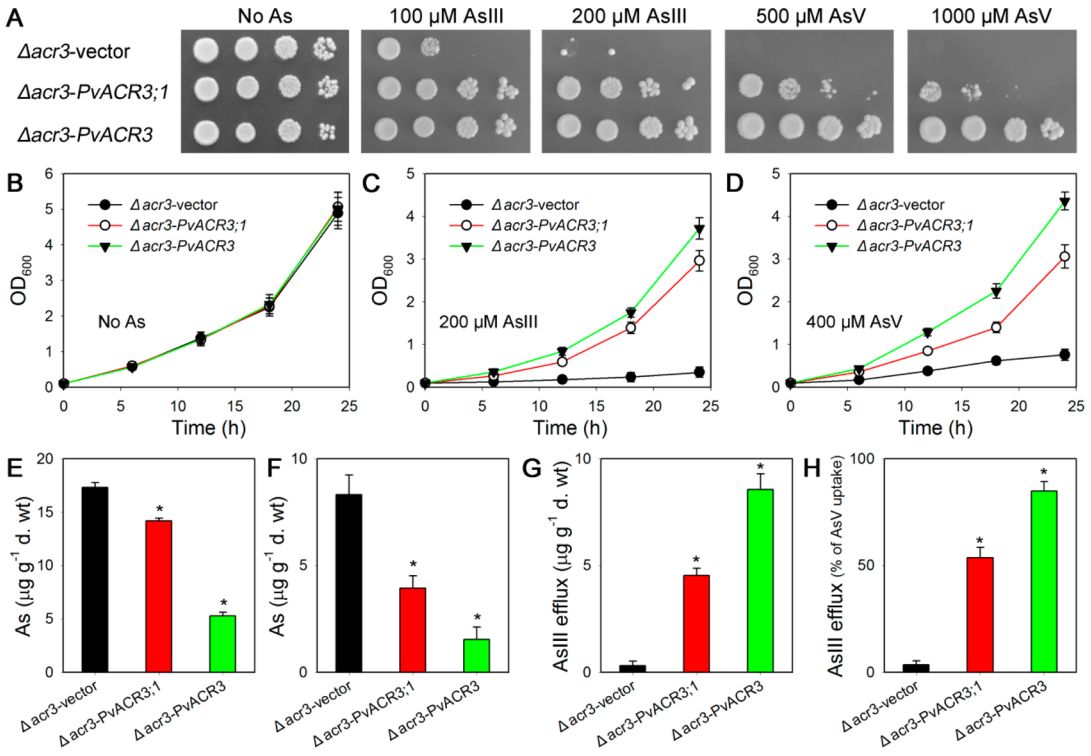

Figure 1. Expressing *PvACR3;1* enhanced Arsenic (As) tolerance in yeast mutant Δ*acr3* by increasing arsenite (AsIII) efflux. *S. cerevisiae* mutant Δ*acr3* was transformed with either vector (Δ*acr3*-vector) or with vector containing *PvACR3;1* CDS (Δ*acr3-PvACR3;1*); Δ*acr3* transformed with *PvACR3* cloned in the same vector was for comparative analyses. (**A**) Yeast cells were diluted in sterile water to an OD_{600} of 1.0, 0.1, 0.01, and 0.001 (from left to right in each section), and then 2 μL Δ*acr3* drops spotted on SD (+2% galactose) plates with or without As. Plates were incubated for 3 d at 30 °C. Drop tests were repeated at least three times with similar results. (**B**–**D**) Growth curves of Δ*acr3* transformed with empty vector (●) and Δ*acr3* expressing *PvACR3;1* (○) or *PvACR3* (▼) at no As (B), 200 μM AsIII (C), and 400 μM AsV (D) for up to 1 d. (**E**-**F**) Arsenic accumulation in Δ*acr3* vector, Δ*acr3-PvACR3;1* and Δ*acr3-PvACR3* yeast cultured in 20 μM AsIII for 2 h (E) and 5 μM AsV for 3 h (F). (**G**-**H**) Arsenite efflux was calculated from the AsV exposure experiments of (F), according to (G) yeast dry weight (d. wt) or (H) as a percentage of yeast AsV uptake. The experiment was performed in triplicate. Asterisk (*) indicates significant difference from the empty vector control based on one-way ANOVA ($P < 0.05$). Error bars = mean ± SEM ($n = 3$).

distilled water three times and tissue sampled to determine As concentrations and species. For analysis of As accumulation in tobaccos under soil cultivation, the soil was spiked with 30 mg/kg AsV and aged for 1 month. Tobacco seeds were germinated and cultivated in As-spiked soils for 4 weeks. The pots were placed at 22 °C with a 16-h light/8-h dark regime and plants were harvested for As determination after 30 d of cultivation.

Total As concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) following Chen et al.[17] Briefly, plant tissues were digested with 50% HNO_3 at 105 °C, following USEPA Method 3050B. For quality assurance and quality control (QA/QC), indium was used as internal standards and was added into the samples, calibration standards, and blanks. During measurement, standard solution at 5 ppb As was measured every 20 samples to monitor the stability of ICP-MS. The check recovery was within 90–110%. In addition, blanks and certified reference material for plant samples (GSB 21, Chinese geological reference materials) were included for quality assurance, which were within expected values. The As concentrations in the spiked soil were also determined according to Han et al.[30]

Arsenic species in the plants were analyzed using high performance liquid chromatography coupled ICP-MS (HPLC-ICP-MS) following Chen et al.[17] Briefly, freeze-dried tobacco plants were ground in mortars with liquid nitrogen and then ultrasonically extracted with 50% methanol. Different As species were separated by an anion exchange column (PRPX100, 10 mm, Hamilton, U.K.) fitted with a guard column (Hamilton, U.K.). Quality assurance was obtained through the blanks, standard curves, and spiked samples.

Subcellular Localization of PvACR3;1. To determine the subcellular localization of PvACR3;1 in plants, PvACR3;1 was fused to GFP by cloning *PvACR3;1* into the 35S promoter cassette of pGFP121 (from pBI121)[16] between *Xho*I and *Kpn*I restriction sites by recombination using the following primers: 5′-ggg act cta gag gat ctc gag ATG GCC GAC AGC ACT CGT TAT GAT C-3′ and 5′-ttc tcc ttt acc cat ggt acc CTA GTA GGG TTC TGC AGG CCA CTT CTT C-3′ (underlining indicating recombination sequences); the constructed plasmid was named pGFP121-*PvACR3;1*. *Agrobacterium* strain C58 was transformed with the binary vector pGFP121-*PvACR3;1* by electroporation. *Agrobacterium* culture was used to transform *A. thaliana* Col-0 by *Agrobacterium*-mediated dip floral transformation. The fluorescence-emitting T2-generation transgenic plants were used to observe PvACR3;1 localization by fluorescence microscopy and laser confocal scanning microscopy (Leica TCS SP8).

RESULTS

Sequence Analysis. Nucleotide Blast showed that the cloned *PvACR3;1* CDS (GenBank accession number: MF740789) varied by 50 nt from reported *PvACR3;1* (GI: 224814385).[23] A comparison of the sequences of predicted

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coding protein is presented in the Supporting Information (SI), which showed a 12 amino acid difference between the current *PvACR3;1* (this study) and the reported *PvACR3;1* (Figure S1).[23]

Yeast Growth Assays. To study the function of *PvACR3;1* gene, full-length CDS was cloned into pAG413GAL-ccdB under control of the GAL1 promoter and expressed in the Δ*acr3* yeast mutant (BY4741 background), which was sensitive to AsIII due to the deletion of yeast AsIII antiporter ScACR3 on the plasma membrane. It was also sensitive to AsV because AsV can be quickly reduced to AsIII by yeast arsenate reductase ACR2.[22,31] Plate growth tests of yeast expressing *PvACR3;1* (Δ*acr3-PvACR3;1*) were performed for 3 d using 100 or 200 μM AsIII, and 500 or 1000 μM AsV (Figure 1A), with yeast carrying empty vector (Δ*acr3*-vector) as control and yeast expressing *PvACR3* (Δ*acr3-PvACR3*) as a comparison.[16] As shown in Figure 1A, expression of *PvACR3;1* induced by galactose enhanced the tolerance of Δ*acr3* to both AsIII and AsV and effectively suppressed As-sensitive phenotype. To confirm the increased As tolerance observed in plate assay, the growth of Δ*acr3* expressing *PvACR3;1* was also monitored in liquid culture for up to 1 d. With no As treatment, Δ*acr3-PvACR3;1* exhibited similar growth to Δ*acr3*-vector (Figure 1B). At 200 μM AsIII or 400 μM AsV, expression of *PvACR3;1* in Δ*acr3* rendered cells more resistant to As compared with vector control (Figure 1CD), indicating that *PvACR3;1* enhanced As tolerance of Δ*acr3*. Arsenic determination showed that expressing *PvACR3;1* significantly reduced As accumulation in the yeast after growing under 20 μM AsIII for 2 h (Figure 1E) or under 5 μM AsV for 3 h (Figure 1F). Considering AsV can be reduced to AsIII in yeast cells, we concluded that the obtained PvACR3;1 was a functional AsIII antiporter, which played an important role in AsIII efflux to the external medium across the yeast plasma membrane, thereby decreasing As accumulation (Figure 1E,F) and increasing As tolerance (Figure 1A–D). However, *PvACR3;1* did not confer as strong As tolerance as reported for *PvACR3* in Δ*acr3* (Figure 1A–D).[19] Although Δ*acr3-PvACR3;1* accumulated significantly lower As in cells than Δ*acr3*-vector, it accumulated higher As in vivo than Δ*acr3-PvACR3* (Figure 1E,F), indicating that PvACR3;1 was less efficient than PvACR3 in mediating AsIII efflux in yeast.

Arsenite can be extruded into external medium by yeast cells following AsV uptake and reduction.[22,32] To further confirm the AsIII efflux behavior of PvACR3;1 in yeast, AsIII in the medium was analyzed after the yeast was grown under 5 μM AsV for 3 h (Figure 1F). The AsIII efflux activity was calculated from the production of AsIII in the solution based on yeast dry weight (Figure 1G) or as a percentage of yeast AsV uptake (Figure 1H). Both results showed that AsIII efflux activity of Δ*acr3-PvACR3;1* was more than 10-fold higher than that of vector control (Figure 1G,H), but lower than that of Δ*acr3-PvACR3*, which further proved that PvACR3;1 mediated AsIII efflux into external medium in yeast but with a relatively lower efficiency than that of PvACR3.

Expressing *PvACR3;1* Gene Decreased Shoot As Accumulation in Plants under AsIII Exposure. *PvACR3;1* gene was transformed into model plant *Arabidopsis* to obtain three homozygous transgenic lines (L5, L7, and L28) expressing *PvACR3;1* under the control of the constitutive CaMV 35S promoter. To investigate the effect of *PvACR3;1* on plant growth and As accumulation, 7-d old seedlings were cultivated for 7 d on plates with various AsIII concentrations (0, 5, or 10 μM). With or without AsIII, the root elongation of the transgenic plants were similar to the wild type (WT) plants, indicating that expressing *PvACR3;1* conferred no significant change in As tolerance (Figure S2A). In addition, at 5 μM AsIII, the shoot and root biomass of the transgenic *Arabidopsis* also showed no significant differences to that of WT (Figure S2BC). Moreover, to further understand the effects of *PvACR3;1* in transgenic *Arabidopsis*, As concentrations in seedlings transferred onto plates with 5 μM AsIII were determined, which represented moderate As concentrations and caused no severe growth inhibition in both WT and transgenic plants. Compared to the WT control, transgenic lines L5, L7, and L28 had 29%, 14%, and 19% higher As concentrations in the roots in 5 μM AsIII treatment after 7 d. In contrast, in the shoots, As accumulation decreased by 55%, 61%, and 60%, respectively, compared to the WT (Figure 2A).

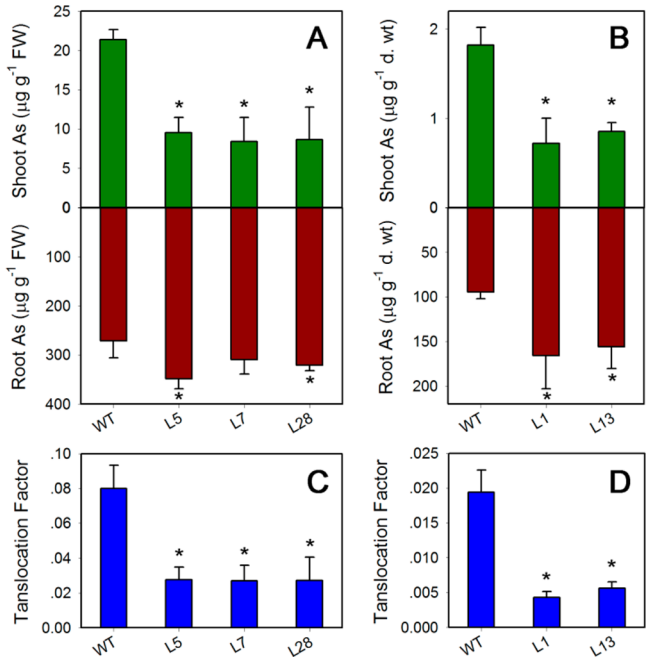

Figure 2. Arsenic (As) accumulation and translocation in *PvACR3;1* transgenic plants under arsenite (AsIII) treatments. (A) Seven-day-old *PvACR3;1* transgenic *Arabidopsis* seedlings (L5, L7 and L28) were transferred onto plates containing 5 μM AsIII and grown for 7 d with WT control. (B) Uniform 30-day-old *PvACR3;1* transgenic tobacco seedlings (L1 and L13) were transferred into 0.2 strength Hoagland-Arnon nutrient solution with 5 μM AsIII and cultivated for 1 d with WT control. Translocation factors (As concentration ratio in shoots to roots) for (C) *Arabidopsis* and (D) tobacco plants. The experiment was performed in triplicate. *Asterisks indicate significant difference from the WT based on one-way ANOVA ($P < 0.05$). Error bars = mean ± SEM ($n = 3$).

In addition, we also transformed the *PvACR3;1* gene into tobacco plants, which is another model plant with higher biomass than *Arabidopsis*. Transgenic tobacco lines L1 and L13 expressing *PvACR3;1* were selected to further analyze the effects of *PvACR3;1* on plants under AsIII treatment. Briefly, uniform 30-d-old tobacco seedlings were transferred into 0.2 strength HNS solution containing 5 μM AsIII and cultivated for 1 d. Similar to transgenic *Arabidopsis*, transgenic tobacco plants also accumulated higher As in the roots and lower As in the shoots compared to the WT control. Specifically, L1 and L13

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showed 75% and 65% higher As in the roots, and 60% and 53% lower As in the shoots (Figure 2B).

In nonhyperaccumulators, most As is often accumulated in the roots, with small proportions of As being translocated to the shoots. This is consistent with low As translocation in *Arabidopsis* (Figure 2C) and tobaccos (Figure 2D). Due to higher root As levels and lower shoot As levels in transgenic plants (Figure 2AB), As translocation dropped by ~66% in transgenic *Arabidopsis* L5, L7, and L28 and by 71−78% in transgenic tobacco L1 and L13, compared to WT controls. These results showed that heterologous expression of *PvACR3;1* decreased AsIII translocation in plants, probably by retaining AsIII in plant roots.

Expressing *PvACR3;1* Gene Decreased AsIII Translocation in Plants under AsV Exposure. In plant roots, AsV can be rapidly reduced to AsIII, making AsIII the dominant As form in plant roots.[5,6] As an AsIII antiporter that alters As partitioning in plants under AsIII treatment, *PvACR3;1* should also play a role in plants under AsV exposure. To test this hypothesis, As accumulation and speciation in transgenic tobacco plant after 5 μM AsV exposure for 1 d were determined. As shown in Figure 3A, compared to the WT, As accumulation was also increased in the roots and decreased in the shoots in L1 and L13.

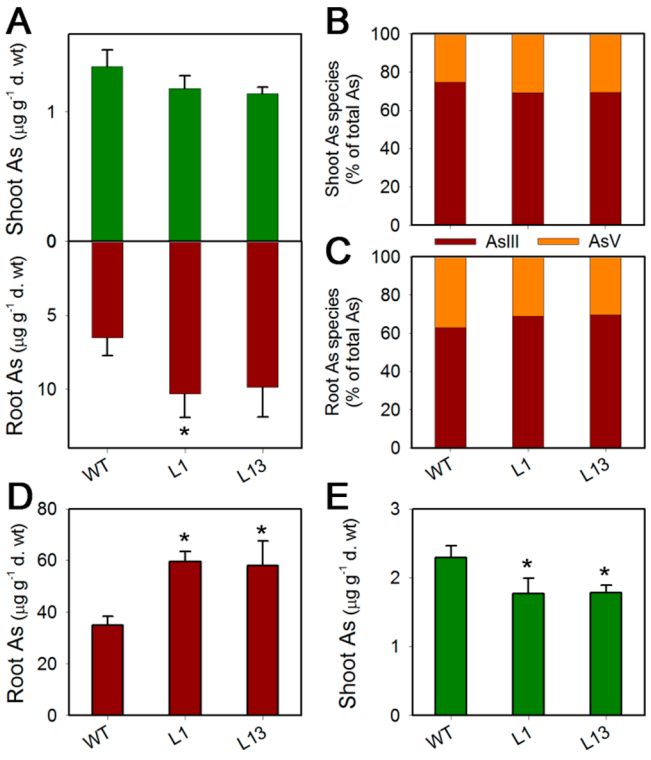

Figure 3. Arsenic (As) accumulation in *PvACR3;1* transgenic tobacco in arsenate (AsV) exposure under soil cultivation. (A) Uniform 4-wk-old *PvACR3;1* transgenic tobacco seedlings (L1 and L13) were transferred into 0.2 strength Hoagland-Arnon nutrient solution with 5 μM AsV and cultivated for 1 d with WT control. Arsenic speciation as a percentage of As in tobacco shoot (B) and root (C) under 5 μM AsV treatment. Arsenic concentrations of the roots (D) and the shoots (E) of tobacco plants of WT, L1, and L13 after growing on soil spiked with 30 mg/kg AsV for 30 d. The experiment was performed in triplicate. Asterisks (*) indicate significant differences from the empty vector control based on one-way ANOVA ($P < 0.05$). Error bars = mean ± SEM.

In both roots and shoots of the WT and transgenic plants, no methylated As species were detected, with AsIII accounting for 63−75% total As (Figure 3BC). However, the As speciation was slightly different between the *PvACR3;1*-expressing lines and WT. In WT shoots, 75% of As was AsIII compared to 69% in L1 and L13, showing a slightly lower AsIII in *PvACR3;1* transgenic lines (Figure 3B). In contrast, AsIII proportion in *PvACR3;1* transgenic lines (69−70%) were higher than that in WT (63%) in the roots (Figure 3C). In *PvACR3;1* transgenic tobacco, increased total As and AsIII in the roots, together with decreased total As and AsIII concentrations in the shoots, further demonstrated that PvACR3;1 decreased AsIII translocation, thereby decreasing As accumulation in the shoots.

Heterologous Expression of *PvACR3;1* Gene Decreased Shoot As in Plants Cultivated in Soil. Because *PvACR3;1* gene decreased As translocation and reduced As accumulation in plant shoots, it may be useful to limit As accumulation in plants for engineering low-As crops. One crucial step in validating the potential of this strategy is to determine whether *PvACR3;1* works effectively in plants cultivated in soils where microorganisms exist and As is bound to soil mainly as AsV. We performed a soil experiment where tobacco seeds were grown for 30 d in soil containing 30 mg/kg AsV, with the plants showing 4−6 leaves. The As contents in the roots and shoots were measured. Notably, the transgenic tobacco lines L1 and L13 accumulated 59.5 and 58.0 $\mu g\ g^{-1}$ DW As in the roots, 70% and 66% higher than WT (Figure 3D). In contrast, L1 and L13 accumulated 1.77 and 1.78 $\mu g\ g^{-1}$ DW As in the shoots, 22% lower than WT (Figure 3E). These data were consistent with the plate experiment of transgenic *Arabidopsis* and hydroponic experiments of transgenic tobaccos, suggesting that *PvACR3;1* can stably affect As partitioning and accumulation in plants under soil experiment. Thus, the *PvACR3;1* transgenic approach in our study may have a potential to limit As accumulation in plant shoots to enhance food safety.

DISCUSSION

Subcellular Localization of PvACR3;1 and Arsenic Accumulation in *PvACR3;1* Transgenic Plants. The subcellular location of AsIII antiporter PvACR3;1 in plants is critical for its physiological function. Thus, vector expressing (C-terminal) PvACR3;1-GFP fusions was transformed into transgenic *Arabidopsis* to visualize GFP fluorescence. *Arabidopsis* root cells have a large vacuole that effectively contours the cell, but clear separation from the cell periphery is visible in the nucleus or in the corners of the rectangular cell periphery.[33] Different from the GFP fluorescence of PvACR3-GFP with the GFP signal completely contouring the periphery of root cells and no gaps being between adjacent cells or separations from the periphery,[16] GFP signal of PvACR3;1-GFP also contoured the cell, but showed clear separation of fluorescence in the adjacent cells (Figure 4A). Moreover, unlike PvACR3-GFP being localized to the plasma membrane, the corresponding fluorescence image of PvACR3;1-GFP showed a clear localization of GFP to the indented region of the rectangular cell, rather than the cell periphery (Figure 4B).

In addition, the PvACR3;1-GFP fusion protein was also subjected to analysis by confocal laser scanning microscopy in the elongated root cells (Figure 4C−E). The bright-field image in Figure 4D showed a clear region of the nucleus, while the corresponding fluorescence image and merged image (Figure 4C−E) showed a clear localization of GFP delineating the

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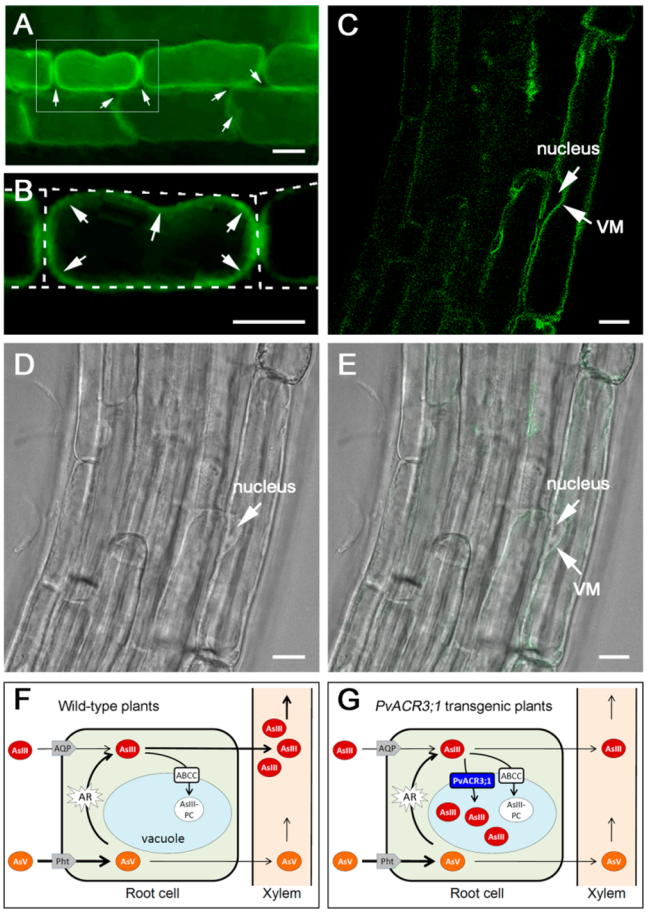

Figure 4. Subcellular localization of PvACR3;1 in plants and the proposed model of As metabolism in *PvACR3;1* transgenic plants. (**A**) The PvACR3;1-GFP fusion protein was expressed in *Arabidopsis* under the CaMV 35S promoter, and GFP fluorescence was observed in the roots by fluorescent microscopy. White arrows indicate gaps of fluorescence between the adjacent cells. (**B**) Regions magnified in white box of (A). White dotted lines represent the rectangular cell periphery and white arrows indicate separations of the fluorescence from the periphery. (**C**–**E**) PvACR3;1-GFP fluorescence image of the elongated root cells (C), the corresponding bright-field image (D) and the merged image of fluorescence and bright-field (E) observed by laser confocal scanning microscopy. White arrows indicate nucleus or vacuolar membrane (VM). Scale bars = 10 μm. The proposed model of As metabolism in (F) wild-type plants and (G) *PvACR3;1* transgenic *Arabidopsis*. AQP = aquaporin; Pht = phosphate transporter; AR = arsenate reductase; PC = phytochelatin; and ABCC = C-type ATP binding cassette transporter.

nucleus at the root cell, unambiguously demonstrating a vacuolar location. Moreover, these results proved that PvACR3;1 was localized to the vacuolar membrane when heterologously expressed in plants, indicating that PvACR3;1 may mediate AsIII sequestration in transgenic plant roots, thus increasing As accumulation in the roots and subsequently decreasing AsIII xylem loading and As translocation to plant shoots (Figure 4FG).

In plants, As can be sequestrated into vacuoles by ABCC transporters as AsIII-PC (Figure 4F), which is an important detoxification mechanism and plays a critical role in As tolerance.[13,14] In this study, *PvACR3;1* also mediated As sequestration in transgenic plants, similar to the effect of ABCC (Figure 4G). *PvACR3;1* did not confer significantly increased As tolerance in transgenic *Arabidopsis* or tobacco, as the transgenic plants exhibited similar growth retardation as WT plants in the presence of As (Figure S2). This was probably because a large proportion of AsIII was chelated with thiols like PCs[15] and transport AsIII into vacuoles may not function as a primary As detoxification strategy in this situation.

Although *PvACR3;1* conferred no significant As tolerance in plants, it markedly altered As partitioning in transgenic plants. Our data showed that, due to increased As retention in the roots, the amount of As being translocated to plant shoots decreased. This was probably because As was translocated from the roots to the shoots as AsIII, so sequestration of AsIII in the roots decreased AsIII loading in the xylem, thereby reducing AsIII translocation and accumulation in the shoots.

Function of *PvACR3;1* Gene in As-Hyperaccumulator *P. vittata*. ACR3 is lost in flowering plants so no *ACR3* exists in the genome of *Arabidopsis*, tobacco, or important cereal crops like rice. Although the function of *PvACR3;1* in transgenic plants is partially revealed, its function in As hyperaccumulation by *P. vittata* is largely unknown. *P. vittata* is the first known As-hyperaccumulator, which is characterized by highly efficient root As uptake, root-to-shoot As translocation, and shoot As accumulation.[24,34] In most plants, high concentrations of As are accumulated in plant roots, with only small amounts being transported to plant shoots.[35] This is consistent with the low translocation factors (ratio of shoot to root concentrations) in plants in our experiments. In contrast, As translocation factor in *P. vittata* is usually >1, often >10.[17,24,34,36] In this study, PvACR3;1 functioned as a firewall to limit As translocation in transgenic plants, conferring As retention in the roots by decreasing As transfer to the shoots, which is unlikely for PvACR3;1 in *P. vittata*.

Indriolo et al. reported two *ACR3* genes, *PvACR3* and *PvACR3;1* in *P. vittata*. PvACR3 is localized to the vacuolar membrane, likely transporting AsIII into the frond vacuoles for sequestration.[23] Although *PvACR3;1* was identified in the same study, the authors did not transform the purified *PvACR3;1* plasmid into yeast cells.[23] Thus, whether *PvACR3;1* could suppress the AsIII-sensitive phenotype of Δ*acr3* yeast and function as an AsIII efflux transporter in yeast is unknown. In our study, we cloned the *PvACR3;1* cDNA, constructed the pAG413GAL-*PvACR3;1* vector, and showed that *PvACR3;1* can complement the arsenic sensitivity phenotype of Δ*acr3* yeast mutant (Figure 1). Our results were consistent with the hypothesis that PvACR3;1 was a functional AsIII antiporter. Considering PvACR3;1 was localized to the vacuolar membrane in transgenic *Arabidopsis* (Figure 4A–E) and As was sequestrated into the fronds to detoxify As in *P. vittata*,[37,38] we speculated that PvACR3;1 may also localize to vacuolar membrane in *P. vittata* to play an important role in frond As sequestration and accumulation.

Comparison of Different *ACR3*s in Transgenic Plants. Although *ACR3* genes are crucial for As detoxification in microbes like yeasts, they are lost in flowering plants.[23] To understand its role in plant As metabolism, *ACR3* genes from different species such as yeast and *P. vittata* have been transformed into different plant species (*Arabidopsis* and rice).[16,39,40]

Transforming yeast *ACR3* gene into *Arabidopsis*, Ali et al. found that ScACR3 localizes to the plasma membrane. Heterologous expression of yeast *ACR3* enhances plant As tolerance, but does not affect As accumulation in different tissues.[39] In addition, Duan et al. transformed *ScACR3* into rice and found that transgenic plants exhibited significant higher

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AsIII efflux than WT control. More importantly, they accumulated lower As in the grains, which can be of significance to engineer low-As rice to enhance food safety.[40] *ACR3* is lost in higher plants, but exists in the fern *P. vittata*. Chen et al. found that PvACR3 localizes to the plasma membrane in *Arabidopsis*, different from its localization in *P. vittata*.[16] Moreover, expressing *PvACR3* greatly increases AsIII efflux into the external medium, thus reducing As accumulation in the roots, thereby increasing As tolerance in transgenic *Arabidopsis*. The shoot As accumulation decreases under low As exposure, but increases significantly under soil cultivation. This is probably because PvACR3 also mediates AsIII transport to the xylem and root-to-shoot AsIII translocation.[16]

Different from previous studies, this study, for the first time, showed ACR3 localization to the vacuolar membrane when heterologously expressed in other plants, rather than the plasma membrane like ScACR3 or PvACR3. While the plasma membrane localization increased AsIII efflux and decreased As accumulation in the roots, the vacuolar localization enhanced AsIII sequestration into vacuoles and elevated As level in the roots. Considering the different subcellular localization and the different behavior of PvACR3;1 from known ACR3s, we believe that PvACR3;1 represents a novel category of ACR3 that may play crucial roles in regulating As accumulation in plants.

Engineering Low-As Crops to Enhance Food Safety. The prevalent As contamination in soil threatens human health through the food-chains, so it is necessary to limit As accumulation in plant shoots.[40,41] During As transport from soil to plants, As can be sequestrated in both roots and shoots. For example, OsABCC1 mediates vacuolar AsIII−PC sequestration in node I, which is critical for reducing As accumulation in rice grains.[14]

It should be noticed that overexpressing transporters for As sequestration in the shoots may also increase shoot As accumulation in plants,[42−45] possibly owing to an increase in shoot As by transporter-mediated sequestration into the vacuoles. For example, yeast ABC transporter YCF1 (Yeast Cd Factor) transports both $Cd(GS)_2$ and $As(GS)_3$ into vacuoles for sequestration,[46,47] and *Arabidopsis* expressing YCF1 accumulates greater amounts of As.[44,45] Guo et al. showed that transgenic lines expressing *YCF1* gene had over 3.4 times amounts of As than that of wild-type under AsIII exposure.[44] LeBlanc et al. also showed that *YCF1* gene overexpression plants contained 26% higher As than WT under 150 μM AsV for 21 d.[45] However, these studies showed As concentrations in whole plants, with no information on As concentrations in the roots and shoots.

In plants, the shoot As content depends on the As taken up from the roots and its translocation from the roots to the shoots. As an AsIII antiporter, PvACR3;1 is not likely to directly change As uptake by the roots, but it probably enables plants to harbor more As in the roots. In our study, when tobacco seedlings were exposed to 5 μM AsIII (Figure 2B) or AsV (Figure 3A), the transgenic plants showed significantly lower shoot As concentrations than that of WT plants. This was because AsIII can be effectively sequestrated and retained in the roots, decreasing AsIII in the root cytoplasm for xylem loading. In this situation, although plant shoots have the capacity to hold higher As, the decreased As translocation played a major role in reducing shoot As accumulation. However, under higher AsIII or AsV, the shoot As accumulation in transgenic tobacco plants may be varied compared to WT control. As shown in Figure S3, when exposed to 20−50 μM AsIII or 50−200 μM AsV, the root As levels were still higher in transgenic lines than WT, consistent with 5 μM AsIII or AsV exposure, suggesting that root AsIII sequestration stably increased As accumulation in the roots. However, when exposed to 50 μM AsIII or 50−200 μM AsV, As concentrations in plant shoots in transgenic lines were comparable to or even higher than that in WT controls. This was probably because *PvACR3;1*-mediated AsIII sequestration had limited effect to decrease AsIII translocation in the roots under high As exposure. Besides, driven by CaMV 35S promoter, *PvACR3;1* was also expressed in the shoots where *PvACR3;1* may also contribute to As vacuolar internalization and enable the shoots to harbor and accumulate more As.

Due to the strong affinity for iron oxides/hydroxides, free AsV concentrations in As-contaminated soils are relatively low. For example, in a highly contaminated soil, AsV in the soil solution is only 2.3 μM.[5] Thus, in our experiment, 5 μM AsIII or AsV treatments can be of environment relevance, suggesting that *PvACR3;1* transgenic approach may significantly decrease As accumulation in plants in As-contaminated soils. In addition, the soil experiment further proved that *PvACR3;1* transgenic approach was effective in decreasing shoot As accumulation in tobacco plants cultivated in moderately As-contaminated soils.

In summary, PvACR3;1 is a critical AsIII antiporter and may play a key role in As metabolism in As-hyperaccumulator *P. vittata*. Heterologous expression of *PvACR3;1* significantly increased As content in plant roots and effectively decreased As accumulation in plant shoots under environmentally relevant As exposure. Hence, this transporter is available for manipulation into food crops to control As accumulation in edible parts. PvACR3;1 localized to the vacuolar membrane in transgenic plants and mediated AsIII sequestration into root vacuoles, and thus decreased AsIII translocation to the shoots, which represents the first report of sequestrating AsIII in plant roots to decrease As translocation. The understanding of the property and the function of PvACR3;1 in plants provides insight to elucidate As hyperaccumulation mechanism of *P. vittata*, and more importantly, provides a potential strategy to breed low-As crops to enhance food safety.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b03369.

Sequence analysis of predicted PvACR3;1 protein, root elongation, and fresh weight of transgenic *Arabidopsis* plants expressing *PvACR3;1* under AsIII treatments, and As accumulation in *PvACR3;1* transgenic tobaccos under different AsIII and AsV treatments (Figures S1−S3) (PDF)

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The authors declare no competing financial interest.

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