Hyperaccumulation of arsenic in the shoots of *Arabidopsis* silenced for arsenate reductase (ACR2)

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Endogenous plant arsenate reductase (ACR) activity converts arsenate to arsenite in roots, immobilizing arsenic below ground. By blocking this activity, we hoped to construct plants that would mobilize more arsenate aboveground. We have identified a single gene in the *Arabidopsis thaliana* genome, *ACR2*, with moderate sequence homology to yeast arsenate reductase. Expression of *ACR2* cDNA in *Escherichia coli* complemented the arsenate-resistant and arsenate-sensitive phenotypes of various bacterial *ars* operon mutants. RNA interference reduced ACR2 protein expression in *Arabidopsis* to as low as 2% of wild-type levels. The various knockdown plant lines were more sensitive to high concentrations of arsenate, but not arsenite, than wild type. The knockdown lines accumulated 10- to 16-fold more arsenic in shoots (350–500 ppm) and retained less arsenic in roots than wild type, when grown on arsenate medium with <8 ppm arsenic. Reducing expression of *ACR2* homologs in tree, shrub, and grass species should play a vital role in the phytoremediation of environmental arsenic contamination.

Escherichia coli ArsC | drinking water | CDC25 | toxicant | arsenic pollution

Environmental arsenic pollution is widely recognized as a global health problem (1) (www.epa.gov/ogwdw/ars/arsenic.html). High levels of arsenic in soil and drinking water have been reported around the world, but the situation is worst in India and Bangladesh, where >400 million people are at risk of arsenic poisoning (2). The World Health Organization predicts that long-term exposure to arsenic could reach epidemic proportions, estimating that 1 in 10 people in the most contaminated areas may ultimately die from diseases related to arsenic poisoning (3). The high financial cost associated with repairing the environmental damage by using physical remediation methods such as excavation and reburial make these technologies unacceptable for cleaning up the vast areas of the planet that need arsenic remediation. As a result, the overwhelming majority of arsenic-contaminated sites are not being cleaned up.

Phytoremediation is the use of plants to clean up environmental pollutants and is considered an important alternative to physical methods for cleaning up arsenic (4). Our objective is to develop a genetics-based arsenic phytoremediation strategy that can be used in any plant species. Plants that hyperaccumulate arsenic to high levels aboveground would be harvested and the arsenic further concentrated by incineration. In previous studies, we engineered model plants expressing a bacterial arsenate reductase (ArsC; EC 1.20.4.1) aboveground and constitutively expressing γ-glutamylcysteine synthetase (5). By reducing arsenate to arsenite in leaves and trapping arsenite in thiol-peptide complexes, these plants accumulate 3-fold more arsenic aboveground than wild type and are also highly tolerant to toxic levels of arsenic. The research described herein extends these observations and attacks a particular problem limiting the engineered phytoremediation of arsenic: its transport from roots to shoots.

The arsenate oxyanions $HAsO_4^{2-}$ and $H_2AsO_4^-$ are the most prevalent forms of arsenic in surface soil, water, and within cells, and these oxyanions contain arsenic in the pentavalent state [As(V)]. Because arsenate is chemically very similar to phosphate, an important plant nutrient, plants take up and translocate arsenate in place of phosphate (6, 7). This plant characteristic should be very useful in phytoremediation strategies, but there is a serious limitation to this system. Most plants appear to have high levels of endogenous arsenate reductase activity, which reduces >95% of the arsenate they take up to arsenite (5, 8). Arsenite, which at neutral pH contains arsenic in the trivalent oxidation state [As(III)], and probably as the acid $HAsO_3^{2-}$, is highly reactive and readily forms As(III)-thiol complexes (9). It appears that in most plants, arsenite is sequestered in roots, preventing it from moving up into stems, leaves, and reproductive organs. Thus, the enzymatic reduction of arsenate to arsenite in roots is a major barrier to engineering plants that will efficiently translocate arsenic to aboveground tissues (5, 8).

Our research has focused on identifying and blocking arsenate reduction activity to engineer plants with enhanced phytoremediation potential. In most, if not all, organisms, arsenate reduction is enzymatically catalyzed (10). The first eukaryotic arsenate reductase to be identified was ScAcr2p from the yeast *Saccharomyces cerevisiae* (11). In this study, we identified an *Arabidopsis thaliana* arsenate reductase 2 gene (*ACR2*) that shares homology with *ScACR2*. *ACR2* complemented the arsenic processing functions of *Escherichia coli* arsenate reductase ($arsC^-$) mutants. We silenced *ACR2* expression in *Arabidopsis*, and the knockdown plants hyperaccumulated large amounts of arsenic aboveground.

Results

Identifying Plant ACR2 Homologs. We searched various plant databases for genes encoding amino acid sequence homologs of the previously described *E. coli* ArsC and yeast ScACR2 arsenate reductases (12–14). We found sequences that were the likely plant homologs of the ScAcr2 protein sequence, but nothing related in overall sequence to ArsC. The gene for the putative *A. thaliana* (Columbia ecotype) homolog, which we designated as the *ACR2* gene, was located on chromosome V (locus At5g03455) and was composed of three exons (Fig. 1*C*). An alignment of the *Arabidopsis* ACR2 protein sequence with that from yeast, *Leishmania,* and several other plant homologs is shown (Fig. 1*A*). The predicted ORF within *ACR2* is 399 bp, encoding a protein of 132 aa with a molecular mass of 14.5 kDa and an estimated basic isoelectric point of pI = 8.9. In pairwise comparisons, ACR2 shares 33% amino acid sequence identity and 42% overall similarity with the 130-residue ScAcr2 protein and 32% identity and 40% overall similarity with the 127-residue LmACR2 protein (15). *Arabidopsis* ACR2, along with the other plant sequences, has the signature amino acid motif (HCX_5R)

Conflict of interest statement: No conflicts declared.

Abbreviations: ACR, arsenate reductase; PTPase, protein-tyrosine phosphatase; RNAi, RNA interference.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. NM_120425 and *Arabidopsis thaliana* chromosome V locus At5g03455).

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required for arsenate reductase activity (10), which is located within the 9-aa active site (underlined, Fig. 1*A*).

The *Arabidopsis*, fungal, and protist ACR2 sequences show homology to a region within the CDC25 superfamily of protein-tyrosine phosphatases (PTPases) that also contains the conserved HCX_5R motif. For example, in pairwise comparisons, the 132-aa-long *Arabidopsis* ACR2 has ≈25–27% identity and 32–33% similarity to a 132-aa region within the ≈600-aa-long sequences of yeast *S. cerevisiae* and *Schizosaccharomyces pombe* CDC25 (Fig. 1). The PTPases are modulators of signal transduction pathways that regulate numerous cell functions. *Arabidopsis* ACR2 has recently been described as a plant CDC25 homolog and shown to act as a PTPase: functioning *in vitro* by activating plant cyclin-dependent kinase activity and hydrolyzing phosphate from artificial substrates (16, 17) and functioning *in vivo* in fission yeast by accelerating the mitotic cell cycle leading to shortened cell length (18). However, a phylogenetic analysis (Fig. 1*B*) showed that *Arabidopsis* ACR2 and the other plant ACR2 homologs were more closely related to known arsenate reductases than to CDC25 PTPases. Parsimony and heuristic trees had nearly indistinguishable topographies (data not shown).

***ACR2* Complements Arsenate Reductase Activity in *E. coli*.** We performed two genetic complementation tests in *E. coli arsC* mutants to determine whether *Arabidopsis ACR2* encodes a protein with arsenate reductase activity. First, we tested the ability of the 132-residue *Arabidopsis ACR2* polypeptide to complement the arsenic sensitivity of *E. coli* strain AW10 (19), which is defective in arsenate reductase activity. In this strain, the entire chromosomal *ars* operon, including the arsenate reductase gene *arsC*, is deleted (Δ*ars*), but the *arsA* and *arsB* genes encoding the bacterial arsenite efflux pump are carried on a plasmid (pArsAB200) (12). Expression of the pump confers resistance to arsenite, but the cells remain sensitive to arsenate, allowing complementation by heterologous arsenate reductases. The *Arabidopsis ACR2* gene was cloned under control of the bacterial promoter to make pACR2/BS (Fig. 1*D*) (see *Methods*). Strain AW10 pArsAB200 was transformed with plasmid pACR2/BS and also with an empty pBS vector or pNA1 (expresses bacterial *arsC*) (5) as negative and positive controls, respectively. A wild-type W3110 strain with an intact *ars* operon was also included as a positive control. The growth kinetics of these strains grown on liquid media with a fixed concentration of arsenate (250 μM), after induction by the *lac* inducer isopropyl β-D-thiogalactoside, are shown in Fig. 2. Strain AW10 with pArsAB200 and pBS was sensitive to arsenate, as expected, because it cannot enzymatically reduce arsenate and make arsenite available to the export pump (20). The *Arabidopsis* sequence in pACR2/BS complemented this phenotype and showed significant resistance to arsenate that was almost equivalent to that conferred by expression of the bacterial ArsC protein from pNA1. Neither strain grew quite as well as the wild-type control (W3110 + pBS). Resistance to arsenate conferred by pACR2/BS is most simply interpreted as caused by the reduction of arsenate to arsenite by ACR2, with subsequent extrusion of arsenite out of the cells by the arsenite export pump.

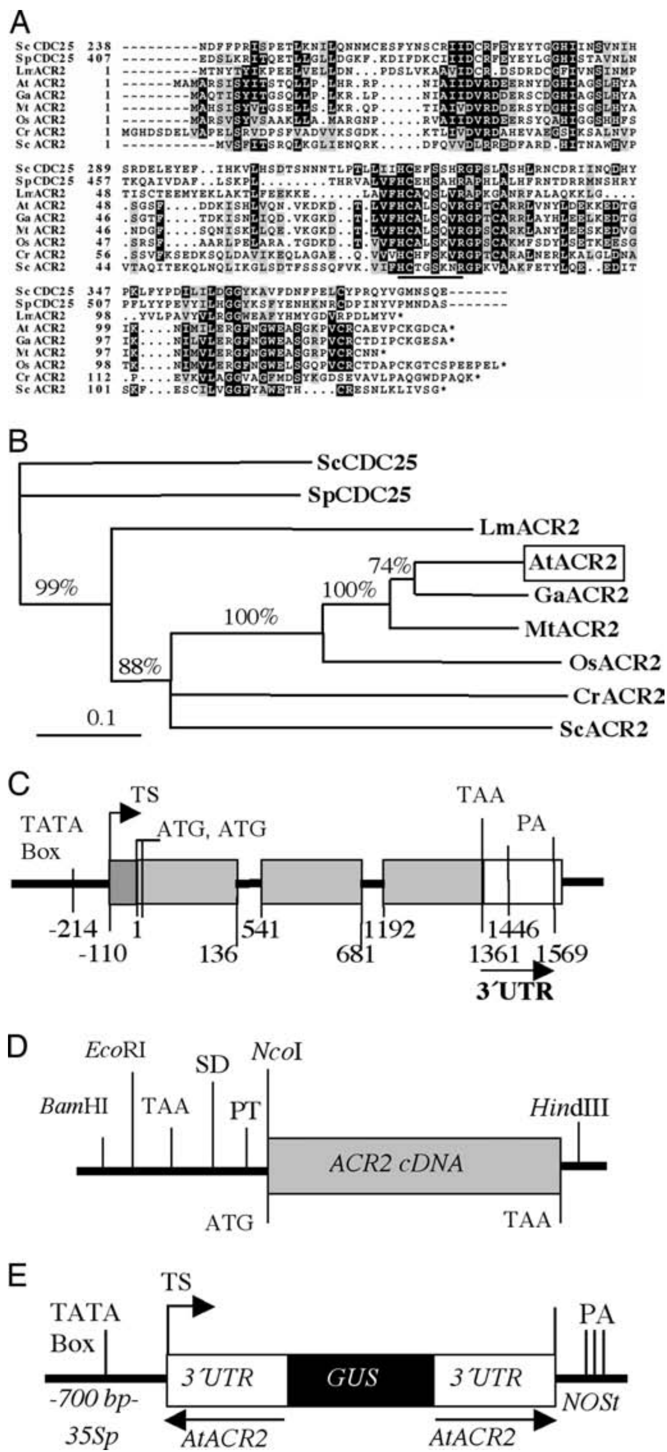

Fig. 1. *Arabidopsis* arsenate reductase ACR2 sequence, phylogeny, and silencing. (*A*) Sequence comparisons with the *Arabidopsis* protein A+ACR2. An alignment of arsenate reductases from *S. cerevisiae* (ScAcr2p, NP_015526) and *Leishmania major* (LmAcr2p, AA573185) (15) with putative reductases from *A. thaliana* (ACR2, NP_568119), *Gossypium arboreum* (GaACR2, AW666950), *Medicago truncatula* (MtACR2, AW20807), *Oryza sativa* (rice) (OsACR2, BE039986), and *Chlamydomonas reinhardtii* (CrACR2, AW661050), and portions of CDC25 sequences from *S. cerevisiae* (ScCDC25, NP_013750) and *S. pombe* (SpCDC25, S62407) is shown. The active site region of these enzymes, which contains HCX_5R, is underlined. (*B*) Protein sequence tree of ACR2-related sequences using the neighbor-joining method (NBJ) prepared in PAUP V. 4.0 with bootstrapping to validate the topography (Sinauer, Sunderland, MA). The scale indicates the fraction of the number of amino acid changes. (*C*) Map of the exon structure of the *Arabidopsis ACR2* gene. The nucleotide positions within the transcript coding sequence are numbered from transcription start site (TS −110). (*D*) The *AtACR2*/*BS* cDNA construct expressed in *E. coli* for complementation studies. (*E*) The RNA interference (RNAi) gene construct, *ACR2Ri,* used to silence gene expression in *Arabidopsis.* TATA box, the characterized sequence specifying the start of transcription; PA, predicted poly(A) addition sites; ATG and TAA, initiation and termination codons; SD, Shine–Dalgarno sequence; PT, plant translation signal; UTR, 5′ and 3′ untranslated regions, white boxes; exons, gray boxes; promoters, introns, and flanking sequences, heavy black lines; β-glucuronidase (GUS) spacer, black box; *ACR2* coding sequences, gray boxes.

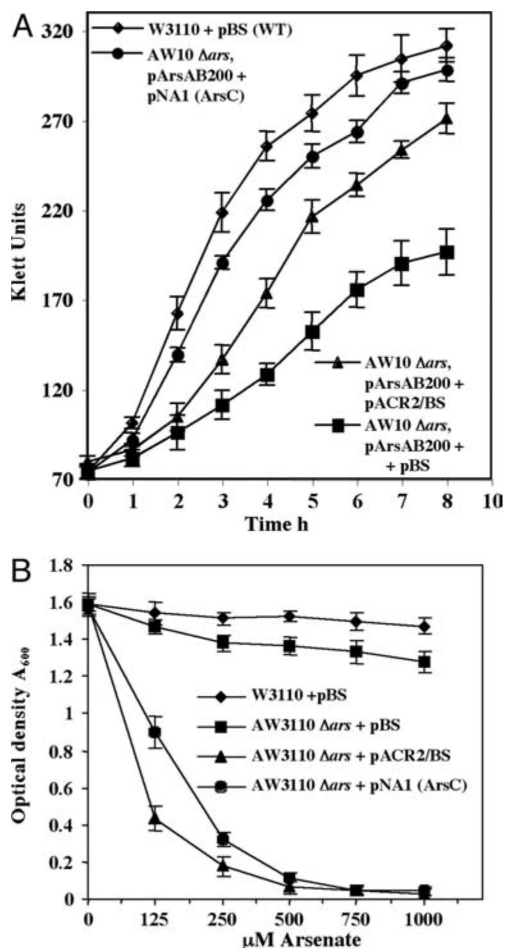

Fig. 2. Complementation of *E. coli ars* operon mutants by *ACR2*. ACR2 complementation assays were performed in *ars* operon deletion mutant strains. (*A*) Arsenic resistance assay. Bacterial strains W3110 + pBS, AW10 pArsAB200 + pBS, AW10 pArsAB200 + pACR2, and AW10 pArsAB200 + pNA1 (ArsC) were grown in the presence of 250 μM sodium arsenate, and cell density was measured in Klett units (green no. 44 filter) as a function of time. (*B*) Arsenic sensitivity assay. W3110 + pBS, AW3110 + pBS, AW3110 + pACR2, and AW3110 + pNA1 were grown on the various concentrations of arsenate indicated. Cell density OD_{600} was assayed after 16 h of growth.

Second, we examined the effect of *ACR2* expression on the growth of an *E. coli* strain, AW3110, from which the entire chromosomal *ars* operon has been deleted (Δ*ars*). AW3110 is the parent strain to AW10 and does not contain a plasmid encoding the ArsA/B pump to eliminate arsenite. AW3110 was transformed with an empty pBS vector, pNA1, or pACR2/BS. Strain W3110 with an intact *ars* operon and pBS served as a wild-type control with a functional *ars* operon. Half-strength LB with increasing concentrations of arsenate (125–1,000 μM) were inoculated with equivalent amounts of each strain, and the final cell density of these strains was determined after 16 h of growth, as shown in Fig. 2*B*. Expression of *Arabidopsis* ACR2 or bacterial ArsC in the AW3110 strain resulted in a significant increase in arsenate sensitivity relative to both wild-type W3110 plus pBS and AW3110 plus pBS. The cells expressing ACR2 or arsC never reached high densities in the presence of even the lowest concentrations of arsenic (125 μM). AW3110 (pBS) was nearly as resistant to arsenate as wild type, because it lacked the endogenous *ArsC* and, thus, did not generate toxic arsenite. The dominant-negative phenotypes for bacterial *ArsC* or *Arabidopsis ACR2* expression were indistinguishable and were consistent with the production of toxic arsenite, which cannot

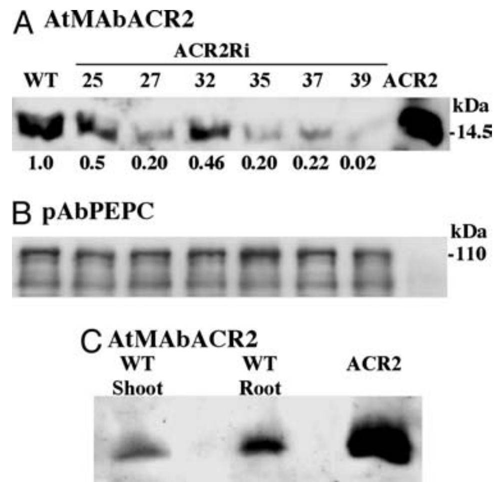

Fig. 3. Analysis of the silencing of ACR2 protein expression in RNAi plant lines. (*A* and *B*) The immunoblot membrane was cut into two strips and reacted separately with ACR2- and phospho*enol*pyruvate carboxylase-specific antisera. (*A*) Western blot analysis of the lower half of the membrane by using the mouse anti-ACR2 antibody, showing the reduction of ACR2 expression in roots of six independent ACR2Ri plant lines compared with wild type (WT) as quantified below each lane. Protein extracts of 14.5-kDa recombinant ACR2 served as positive control (right lane). (*B*) Upper portion of membrane reacted with phospho*enol*pyruvate carboxylase antisera. (*C*) ACR2 was observed in both shoots and roots.

be eliminated from the AW3110 strain lacking an arsenite export pump. These data strongly suggest that the *Arabidopsis ACR2* gene encodes an arsenate reductase.

Generating Plant Lines Knocked Down for ACR2 Activity. We used RNAi to knock down *ACR2* expression. The RNAi gene construct *ACR2Ri* (Fig. 1*E*) targeted ≈200 bp of the 3′ UTR of the *ACR2* transcript for degradation and was expressed under the control of a constitutive promoter in transgenic *A. thaliana*. Six RNAi lines with single transgene insertions were designated as Ri25, Ri27, Ri32, Ri35, Ri37, and Ri39. The T_2 generation transgenic seeds and plants did not show any phenotypic differences from nontransformed *Arabidopsis* when grown on regular plant media or soil.

Immunoblot analysis of root tissues by using an ACR2-specific antibody showed a significant decrease in the levels of the 14.5-kDa ACR2 protein in all of the knockdown RNAi lines compared with wild-type plants (Fig. 3*A*). Specifically, the RNAi lines contained from 2% to 50% of wild-type protein levels. The levels of the 110-kDa phospho*enol*pyruvate (PEP) carboxylase (pAbPEPC) polypeptide served as a loading and protein transfer control (Fig. 3*B*). ACR2 protein was also detected in shoot tissues of wild-type plants (Fig. 3*C*), although the levels of protein were lower than in roots. Microarray analyses of *Arabidopsis* transcript levels suggest that *ACR2* mRNA is constitutively expressed at low levels in all plant organs (21).

Growth of the ACR2 RNAi Knockdown Lines in the Presence of Arsenate and Arsenite. *Arabidopsis* wild type and the six independent ACR2Ri knockdown lines assayed by Western blotting (Fig. 3) were tested for arsenic sensitivity by germination on medium containing various arsenate concentrations, as shown for line ACR2Ri39 (Fig. 4*A*). Concentrations of arsenate of 100 μM or less had minimal effects on the growth of either wild-type plants or the RNAi lines. The RNAi lines were significantly more sensitive to arsenate in comparison with wild type at 150 μM arsenate, as summarized for all six lines combined (Fig. 4*B*). The ACR2Ri lines germinated as well as wild type, but, after 3 weeks of growth, the knockdown plants attained 5- to 6-fold less fresh weight than wild-type controls. There

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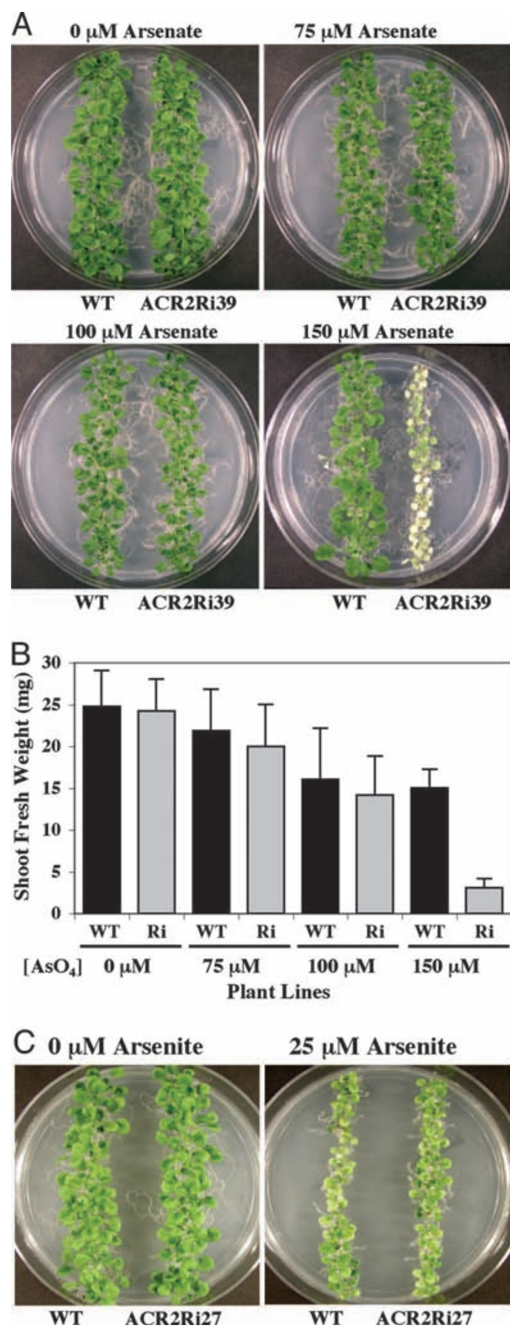

Fig. 4. *Arabidopsis* RNAi plant lines are sensitive to arsenate but not arsenite. (*A*) Arsenate sensitivity of the knockdown line ACR2Ri39 expressing the *ACR2Ri* construct were compared with wild type (WT) on medium with 0, 75, 100, and 150 μM sodium arsenate. Similar results were obtained for all six lines (ACR2Ri25, -27, -32, -35, -37, and -39). (*B*) Summary of the comparative growth inhibition between the RNAi lines and the wild-type plants grown as shown in *A*. The mean and SE are from three replicates of >40 seedlings for wild type and 30 each of the six *ACR2Ri* lines (ACR2Ri25, -27, -32, -35, -37, and -39) combined for the ACR2Ri samples. On 150 μM arsenate, biomass accumulation was significantly lower for each of the RNAi lines as compared with wild type ($P < 0.001$). (*C*) Growth of the RNAi knockdown lines expressing the *ACR2Ri* construct compared with wild type on 25 μM sodium arsenite. (*A–C*) Seeds were germinated and plants were grown for 3 weeks on half-strength MS medium (23) with 16-h days at 22°C, as described in Dhankher *et al.* (29).

was no significant difference in fresh weight between transgenic lines and wild-type plants when grown on media not supplemented with arsenate (Fig. 4 *A* and *C Left*).

If the *Arabidopsis* ACR2Ri knockdown lines are arsenic sensitive because of a lack of arsenate reduction to arsenite, there should be no difference in their sensitivity to arsenite. Both transgenic and wild-type plants were grown on a concentration of arsenite [25 μM As(III)] that significantly inhibited the growth of wild type, as shown for one line in Fig. 4*C*. Both wild type and the ACR2 RNAi knockdown lines were equally sensitive to arsenite, and there were no obvious phenotypic differences. These results are consistent with *Arabidopsis* ACR2 functioning as an arsenate reductase.

Hyperaccumulation of Arsenic in the Shoots of the ACR2 Knockdown Lines. Our model predicts that arsenate is the most mobile form of arsenic in the majority of plant species and that arsenite stays trapped in roots. Thus, if these RNAi lines enzymatically reduced arsenate less efficiently than wild type because of lower ACR2 enzyme levels, they should transport more arsenate to shoots. The ACR2Ri knockdown lines showed significantly higher concentrations of arsenic in their shoots and retained slightly less arsenic in their roots than wild type (Fig. 5 *A* and *B*), when grown on 100 μM arsenate. At this concentration of toxicant, the RNA interference (RNAi) lines were not significantly inhibited in growth relative to wild type. Quantitative assays showed that these RNAi knockdown lines accumulated 10- to 16-fold more arsenic in shoots (350–500 ppm arsenic) compared with wild-type controls, which accumulated only 30 ppm arsenic. In several repetitions of this experiment with 75 and 100 μM arsenate in the medium, all of the RNAi lines tested accumulated between 6 and 20 times higher levels of arsenic than the wild type (data not shown). Whereas wild-type plants have shoot-arsenic concentrations that are only 1% of their root levels, the RNAi lines have shoot concentrations that are ≈25% of their root levels (Fig. 5). Clearly, ACR2 activity plays a significant role in blocking long-distance arsenic transport and accumulation in wild-type plants.

When plants were grown with 25 μM arsenite in the medium, as illustrated in Fig. 4*C*, there was no difference in the arsenic accumulation of the RNAi lines (Fig. 5*C*). These results also suggest that the substrate for ACR2 protein is arsenate and not arsenite and that blocking ACR2 function enhances arsenate transport from roots to shoots but does not affect endogenous arsenite uptake and transport.

ACR2 Knockdown Lines Accumulated Less Phosphorus in the Presence of Arsenate, but Not Arsenite. Competition between arsenate and phosphate has been shown in several arsenic uptake studies (7, 22, 23). We analyzed the accumulation of total phosphorus in plants grown with the normal amount of phosphate (625 μM) in half-strength MS medium, but we included 100 μM arsenate. The ACR2 knockdown plants accumulated 2- to 3-fold less phosphorus in shoots compared with wild-type plants in response to arsenate exposure (Fig. 5*D*), and some lines retained slightly less phosphorus in roots (Fig. 5*E*). When grown with 25 μM arsenite in the medium, there was no difference between phosphorus accumulation in the wild-type and RNAi lines (Fig. 5*F*). When no arsenate was added to growth media, there was again no difference in phosphorus accumulation between wild-type and the ACR2-deficient lines, with all lines accumulating ≈8,000 ppm phosphorus in shoots (data not shown).

Plant lines ACR2Ri25 and ACR2Ri32, which had only moderate reductions in ACR2 protein levels compared with the other four lines examined (Fig. 3*A*), showed only slight differences from wild type in the root accumulation of arsenic and phosphorus (Fig. 5 *B* and *E*). The ACR2Ri25 line also showed less accumulation of arsenic in shoots than the other lines, a finding that was consistent with its moderate RNAi phenotype (Figs. 3*A* and 5*A*). Further experimentation would be necessary to explain why line ACR2Ri32 accumulated as much arsenic in shoots as the other stronger epialleles with less ACR2 protein.

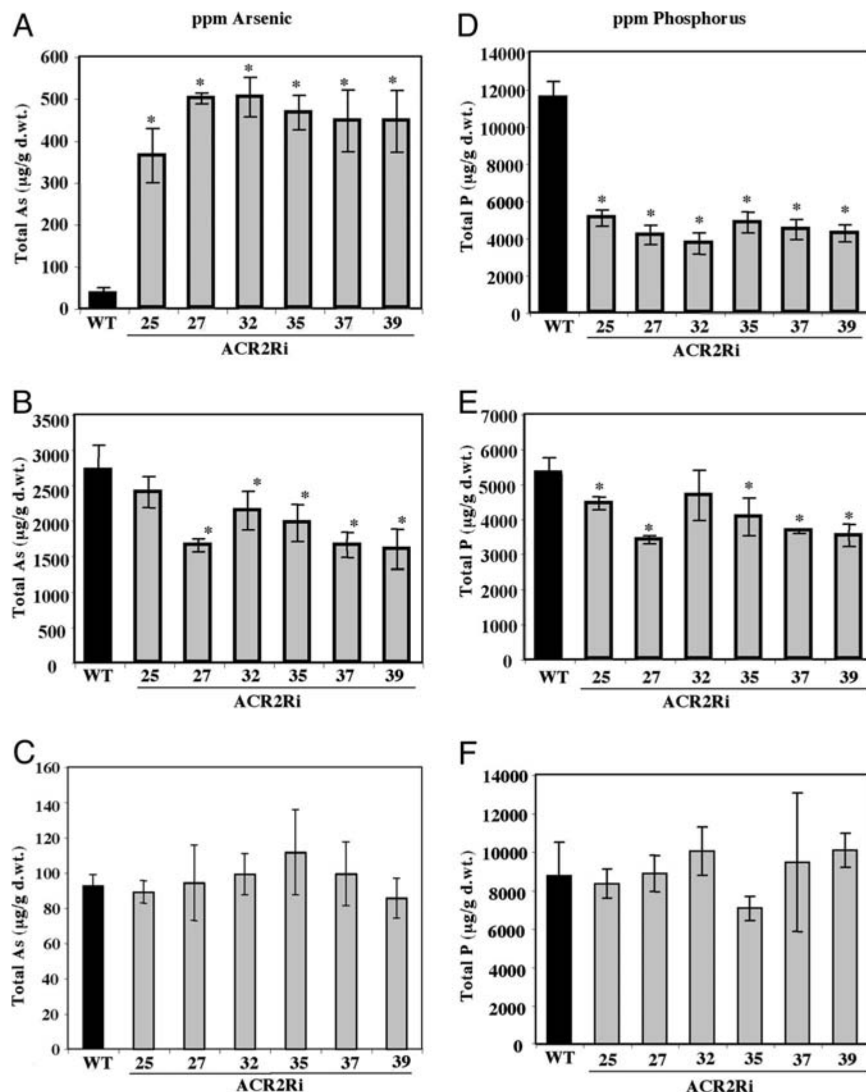

Fig. 5. Arsenic and phosphorus accumulation in ACR2 knockdown lines. Total arsenic accumulation per gram dry weight (d.wt.) was determined in shoots (*A* and *C*) and roots (*B*) of the wild type (WT) and six *ACR2Ri* transgenic *Arabidopsis* lines when seedlings were grown on 100 μM sodium arsenate (*A* and *B*) or when grown on 25 μM sodium arsenite (*C*). Total phosphorus accumulation was determined in shoots (*D* and *F*) and roots (*E*) of the wild type and six *ACR2Ri* transgenic *Arabidopsis* lines when seedlings were grown on 100 μM sodium arsenate (*D* and *E*) or on 25 μM sodium arsenite (*F*). Inductively coupled plasma mass spectrometry assays were performed on 3-week-old plants grown on 0.5$\times$ MS medium supplemented with arsenate or arsenite. The SE values were determined for three replicates of 50 seedlings for each line. An asterisk indicates a significant difference ($P < 0.001$) in arsenic or phosphorus accumulation as compared with wild type.

Discussion

ACR2 Functions as an Arsenate Reductase. *Arabidopsis* ACR2 (CDC25) overexpression in *S. pombe* alters cell length apparently by means of a PTPase activation of cyclin-dependent kinases; however, its functional role as a PTPase *in vivo* in plants has not yet been demonstrated (16, 18). We present several lines of evidence herein that the *Arabidopsis ACR2* gene encodes a functional arsenate reductase. First, ACR2 is more closely related in both size and sequence to *S. cerevisiae* arsenate reductase than it is to *S. cerevisiae* or *S. pombe* CDC25. Second, *Arabidopsis ACR2* expression complemented the arsenate-sensitive phenotype of *E. coli*, which was deficient in the arsenate reductase *arsC* gene, by restoring arsenate resistance. *ACR2* expression alone, like bacterial *ArsC* expression alone, also created the expected arsenate-sensitive phenotype in an *E. coli* strain deficient in the entire *ars* operon. Third, plants with their ACR2 protein levels knocked down by RNAi were hypersensitive to high concentrations of arsenate but were not more sensitive to arsenite, similar to yeast deficient in the *ScACR2* gene (14, 24). Fourth, knocking down ACR2 in plants facilitated order-of-magnitude increases in the aboveground accumulation of arsenic when these plants were exposed to arsenate but not when they were exposed to arsenite. When exposed to moderate levels of arsenate (7.5 ppm arsenic), most of the knockdown lines accumulated arsenic to 500 ppm of the shoot dry weight compared with 30 ppm in wild-type shoots. Fifth, phosphate accumulation in shoots was reduced in the RNAi knockdown plants when they were exposed to arsenate but not when they were exposed to arsenite. When arsenate reductase activity is blocked, higher intercellular arsenate concentrations should both compete for and inhibit phosphate-transport machinery. In contrast, arsenite is not a phosphate analogue and should not have any such inhibitor activity. In other words, the activity of long-distance phosphate transport was strongly affected by ACR2 activity, and there was competition between arsenate and phosphate for this transport system. All of these phenotypes were consistent with ACR2 functioning as an arsenate reductase and cannot be explained by the dual activity as a PTPase. There were no plant phenotypes associated with the knockdown of ACR2 that were not dependent on the presence of arsenate. While this manuscript was in preparation, Duan *et al.* (25) demonstrated that a mutant of *Arabidopsis ACR2* lost detectable *in vitro* reductase activity as assayed in plant protein extracts.

The most parsimonious interpretation of arsenic accumulation data is that the knockdown lines were unable to reduce arsenate to arsenite efficiently, and thus, more arsenate moved into aboveground plant tissues as an analogue of phosphate. It is logical to propose that plants evolved arsenate reductase activities precisely to prevent the competition of arsenate for phosphate transporters and to allow normal translocation of phosphate on arsenic-contaminated soils.

Engineering Plants for the Phytoremediation of Arsenic. Part of our strategy for engineering an aboveground hyperaccumulator of arsenic is based on blocking the endogenous reduction of arsenate to arsenite and thus allowing more efficient arsenate transport into aboveground organs (26). The arsenic phenotypes of the plant lines silenced for *ACR2* expression are consistent with ACR2 playing a major role in the reduction of arsenate to arsenite and arsenic retention in roots. Here, we have shown that silencing the activity of *ACR2* by RNAi resulted in long-distance translocation of 10- to 16-fold more arsenic aboveground. Our prior studies showed that *Arabidopsis* expressing *E. coli* ArsC and γ-glutamylcysteine synthetase accumulated 3-fold more arsenic aboveground and were more tolerant to arsenic than wild-type plants (5). Combining a knockdown of ACR2 with the expression of these two bacterial genes has the potential to generate a super hyperaccumulator with normal plant growth and 30- to 40-fold higher levels of aboveground arsenic. Such plants could contribute significantly to the remediation of arsenic pollution. It should be possible to silence homologues of ACR2 in field-adapted grasses, shrubs, and trees suited to the phytoremediation of arsenic-contaminated sites and water resources.

Methods

Bacterial and Plant Expression of *ACR2* Gene Sequences. The 132-codon *ACR2* cDNA was amplified by PCR using a sense primer, 5′-TACGTCGGATCCTAAGGAGGATAGACCATGGCGATGGCGAGAAGCAT-3′, and an antisense primer, 5′-TAGGTCCTCGAGTTAGGCGCAATCGCCCTTGCAAGGAACCTCTGCACA-3′, from an *Arabidopsis* flower cDNA library (27). The *ACR2* sequence was cloned into BamHI–XhoI replacement region of pBluescript II SK (Stratagene) to make a bacterial expression plasmid pACR2/BS (Fig. 1*D*). This construct was transformed into the two *E. coli* strains AW10 (pArsAB200) and AW3110. The same *E. coli* strains were also transformed with empty plasmid pBS to serve as negative controls.

To silence *ACR2* activity, we made an RNAi construct in which the 3′ UTR sequence from *ACR2* (207 nt after the stop codon) was assembled in reverse and forward orientations, respectively, flanking a 1,000-nt β-glucuronidase spacer region to make a stem–loop RNA transcript (Fig. 1*E*) by using the methods described in ref. 28. The ≈1.4-kb PCR fragment was cloned under control of the cauliflower mosaic virus 35S promoter and nitric-oxide synthase terminator in a binary vector described in ref. 29 to make *ACR2Ri*. This construct was introduced into *A. thaliana* (Columbia ecotype) by *Agrobacterium*-mediated transformation by using the vacuum infiltration procedure (30). The T_1 generation seeds were screened for a linked kanamycin resistance marker, and the first six lines that segregated for a single insertion were selected for further study.

Arsenate Resistance and Sensitivity Assays in Bacteria. Attempts to complement yeast *ScAcr2* mutants with the *Arabidopsis ACR2* were unsuccessful; therefore assays of the plant arsenate reductase gene activity were performed in *E. coli arsC* mutant backgrounds. Liquid culture growth assays for *E. coli* strains (Fig. 2) were performed as described in Mukhopadhyay and Rosen (24). The data reported are the average results of three replicates. Strains were grown overnight at 37°C in Luria–Bertani medium (LB) with appropriate antibiotics and isopropyl β-D-thiogalactoside (IPTG). For the time-dependent liquid growth curve assays (Fig. 2), the cultures were diluted 100-fold into half-strength LB and grown for various time periods indicated in the presence of final concentrations of 100 mg/liter ampicillin, 50 mg/liter kanamycin, 1 mM IPTG, and 250 μM sodium arsenate. Cell density was measured as Klett units at 1-h intervals. Strains AW10 with pArsAB200 and W3100 are described in Liu *et al.* (12) and AW3110 in Carlin *et al.* (20).

Immunoblot Assays of ACR2 Protein. Shoot or root tissues were ground in liquid nitrogen, suspended in extraction buffer (31), and pelleted at 10,000 × *g*. The pellet was reextracted with sample buffer (32) and centrifuged again at 10,000 × *g*, and the supernatant contained the ACR2 protein. An equal amount (10 μg) of total protein from each sample was resolved on a 12% (wt/vol) polyacrylamide gel by SDS/PAGE and blotted to membrane. Equal loading of each sample was first assured by Coomassie staining of samples on a separate gel (data not shown). Western blots of plant extracts were developed as described in Bizily *et al.* (33), by using polyclonal sera as described for phospho*enol*pyruvate carboxylase (33) and mouse antisera to ACR2 followed by horseradish peroxidase-conjugated goat anti-mouse antisera (Sigma) and enhancement by using an enhanced chemiluminescence kit from Amersham Pharmacia following the manufacturer's instructions. The mouse antibody was prepared against a 4-fold multiple antigenic peptide containing the C-terminal 25-aa region of ACR2 by using a method described in ref. 34.

Arsenic and Phosphorus Accumulation in Plants. The ACR2 RNAi knockdown lines were grown on half-strength Murashige and Skoog (MS) medium (35) containing 100 μM sodium arsenate (7.5 ppm elemental arsenic) for 3 weeks. The shoots and roots from these plants were harvested, washed, and extracted for inductively coupled plasma mass spectrometry determination of total arsenic or phosphorus as described in refs. 5 and 36.

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