An Arsenate-activated Glutaredoxin from the Arsenic Hyperaccumulator Fern *Pteris vittata* L. Regulates Intracellular Arsenite*

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To elucidate the mechanisms of arsenic resistance in the arsenic hyperaccumulator fern *Pteris vittata* L., a cDNA for a glutaredoxin (Grx) Pv5–6 was isolated from a frond expression cDNA library based on the ability of the cDNA to increase arsenic resistance in *Escherichia coli*. The deduced amino acid sequence of Pv5–6 showed high homology with an *Arabidopsis* chloroplastic Grx and contained two CXXS putative catalytic motifs. Purified recombinant Pv5–6 exhibited glutaredoxin activity that was increased 1.6-fold by 10 mM arsenate. Site-specific mutation of Cys67 to Ala67 resulted in the loss of both GRX activity and arsenic resistance. PvGrx5 was expressed in *E. coli* mutants in which the arsenic resistance genes of the *ars* operon were deleted (strain AW3110), a deletion of the gene for the ArsC arsenate reductase (strain WC3110), and a strain in which the *ars* operon was deleted and the gene for the GlpF aquaglyceroporin was disrupted (strain OSBR1). Expression of PvGrx5 increased arsenic tolerance in strains AW3110 and WC3110, but not in OSBR1, suggesting that PvGrx5 had a role in cellular arsenic resistance independent of the *ars* operon genes but dependent on GlpF. AW3110 cells expressing PvGrx5 had significantly lower levels of arsenite when compared with vector controls when cultured in medium containing 2.5 mM arsenate. Our results are consistent with PvGrx5 having a role in regulating intracellular arsenite levels, by either directly or indirectly modulating the aquaglyceroporin. To our knowledge, PvGrx5 is the first plant Grx implicated in arsenic metabolism.

Arsenic is a toxic element and is widely distributed in the environment from both natural and anthropogenic sources. Inorganic arsenic, including the oxidized form arsenate (As(V)) and the reduced form arsenite (As(III)), are the most prevalent in the environment (1). As a consequence of its ubiquity, arsenic ranks first on the Environmental Protection Agency Superfund List (U.S. Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Priority List of Hazardous Substances. It is a carcinogen that has been linked to cancers of bladder, liver, kidney, and lungs (2). Millions of people in many parts of the world are affected by arsenic contamination in drinking water (2).

Ma et al. (3) reported that Chinese brake fern *Pteris vittata* was resistant to arsenic and was capable of hyperaccumulating a large amount of arsenic in its fronds (3). Although most plant species are severely affected by exposure to as low as 10 mg-liter⁻¹ arsenic in the medium, *P. vittata* and related ferns tolerate up to 1000 mg-liter⁻¹ arsenic in the medium, with fronds accumulating the metalloid up to 2.3% of their dry weight (3–6). Hence, the potential of using *P. vittata* to phytoremediate arsenic-contaminated soil and water has been evaluated (7, 8).

Despite the extraordinary ability of *P. vittata* in arsenic accumulation, the mechanisms underlying arsenic resistance and hyperaccumulation in this fern are not clear. Understanding arsenic metabolism in this fern is essential for fully harnessing its phytoremediation capacity. Its genes are potentially valuable for building transgenic plants and microorganisms for arsenic remediation. Novel enzymatic transformations of arsenic may also be of interest for improving arsenic cancer therapy because arsenic trioxide has been used to treat certain leukemias (9).

Arsenate is taken up by *P. vittata* via phosphate uptake systems (4, 10), transported via xylem (11), reduced to arsenite in the fronds (12, 13), and likely stored in the vacuoles (14). When compared with an arsenic-sensitive fern *Pteris ensiformis*, *P. vittata* had significantly greater tolerance to oxidative stress, greater levels of reduced GSH, and antioxidant enzymes (15, 16). However, the specific roles of GSH in arsenic tolerance of this fern are still unknown. Using a functional cloning method, we identified *P. vittata* frond cDNAs involved in arsenic tolerance (13). Here we report the characterization of *P. vittata* cDNA encoding a glutaredoxin (Grx)² involved in arsenic resistance. Grxs are ubiquitous oxidoreductases of the thioredoxin family, which catalyze dithiol-disulfide exchange reactions or reduce protein-mixed glutathione disulfides. They use

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) EF052272.

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2 The abbreviations used are: Grx, glutaredoxin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MES, 4-morpholinoethanesulfonic acid; HED, hydroxyethyl disulfide; r, recombinant; Pv, *P. vittata*; At, *A. thaliana*; Ec, *E. coli*; PICOT-HD, protein kinase C-interacting cousin of thioredoxin homology domain; CAX,cation exchanger.
the reducing power of GSH to catalyze disulfide reactions in the presence of NADPH-dependent GSH reductase (17, 18). Grx can reduce protein mixed disulfides through a dithiol mechanism by utilizing two cysteine residues from the active site, or Grxs can deglutathionylate GSH-protein mixed disulfides through monothiol mechanism by using a single cysteine at the active site of the Grx molecule.

In *Escherichia coli*, three bicysteinic Grxs, and in yeast, two bicysteinic and three mono cysteinic Grxs have been characterized, respectively (19), but plant genomes contain many Grxs. In *Arabidopsis*, there are at least 31 isoforms (20). However, the functional roles for the multitude of Grxs in plants are enigmatic (20), but plant genomes contain many Grxs. Although a bacterial Grx (Grx2) is known to be a functional roles for the multitude of Grxs in plants are enigmatic, respectively (19), but plant genomes contain many Grxs.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Bacterial Strains**—All chemicals were obtained from Sigma. Table 1 lists *E. coli* strains used and their sources (26–28). The strains were grown in Luria-Bertani (LB) medium in a shaker incubator set at 200 rpm and 37 °C with appropriate antibiotics (carbenicillin 50 mg liter⁻¹). The strains were grown in LB agar with 5 mM MES-KOH, pH 6.5, and 0.1 mM isopropyl β-D-thiogalactoside (IPTG), containing different levels of sodium arsenate or sodium arsenite and 50 mg liter⁻¹ carbencillin. The cultures, inoculated with late log phase cells, were incubated at 37 °C, 200 rpm, and culture turbidity was measured after 18 h. Similar assays and conditions were used when *E. coli* were tested for tolerance to hydrogen peroxide, methyl violagen, and cadmium chloride.

**Sequence Analyses**—BLAST analysis (29) was used to identify sequences homologous to Pv5–6 deduced amino acid sequence. Multiple sequence comparisons were done using Clustal X (30) and HHpred (31).

**Expression and Purification of Recombinant Protein**—Pv5–6 cDNA (647 bp, including 552 bp of protein coding region and a stop codon, followed by 92 bp of 3′-untranslated region) was amplified using forward and reverse primers 5′-CCAGCCTGATGCTGCCAC-3′ and 5′-GAGCTCACCTGGAAATTGCAGCTACC-3′, respectively. The PCR product was cloned into Ncol and SstI restriction sites of pET30a expression vector to obtain pET30a-Pv5–6, verified by sequencing and transformed into BL21 (DE3). This construct expressed a fusion protein with an N-terminal hexa-histidine tag. Recombinants were grown at 37 °C to 0.8 A₆₀₀, and expression was induced with 0.1 mM IPTG for 4 h. The cells from 500 ml were suspended in bug bustert digestion medium (Novagen, San Diego, CA) and incubated for 2 h on ice. After centrifugation at 10,000 × g for 10 min, the supernatant was loaded on pre-equilibrated Sephadex-G50 column (150 × 1 cm). The column was eluted with 10 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA and 0.1 mM dithiorthreitol. The fractions (5 ml each) were collected with the flow rate of 30 ml h⁻¹. The active fractions were pooled, concentrated using Centricon column-20 (Millipore, Bedford, MA), and loaded onto 10 ml of ProBond resin (Invitrogen) nickel affinity column and step-eluted with 100, 150, and 250 mM imidazole. Total protein was estimated using the Bradford method using bovine serum albumin as a standard (32).

**Thiol Determination and N Termimus Sequencing**—The content of Cys-residues was determined in purified recombinant protein, using Ellman’s reagent following a spectrophotometric method (33). Enterokinase-digested purified protein was separated using SDS-PAGE and blotted onto polyvinylidene difluoride membrane and stained with Coomassie Brilliant Blue. The N terminus was sequenced by Edman degradation using a Procise protein sequencer 494-HT (Applied Biosystems, Foster City, CA).

**Glutaredoxin and Arsenate Reductase Assays**—Grx activity was determined with a coupled enzymatic reaction as described previously (34) by measuring the reduction of 2-hydroxyethyl disulfide (HED) in the presence of NADPH and glutathione reductase. The decrease in NADPH was monitored at 340 nm.

**TABLE 1**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| XL-1 Blue | *endA1, gyrA96, hisD58, lacZΔM15, proAB, thi-1, thl-1 | BD Biosciences |
| AW3110 | K12 F′ IN(rrnD-rrnE) Δars-cam | Ref. 26 |
| WC3110 | K12 F′ IN(rrnD-rrnE) ΔarsG | Ref. 27 |
| OS8R1 | AW3110 gpt::TriplEx–Trp | Ref. 28 |
| BL21 (DE3) | F′ ampr lacIq lacZ M15 | Novagen |

**Glutaredoxin and Arsenate Resistance**

**In vitro Mutagenesis**—In vitro mutagenesis of the Pv5–6 plasmid was carried out using the Gene Tailor™ site-directed mutagenesis system, as per the manufacturer’s instructions. Presumptive Grx active sites were mutated by converting the Cys⁶⁷ to Ala⁶⁷ or Cys¹⁰₈ to Ala¹⁰₈. The forward and reverse primers used were 5′-GGTACCAAGTTGGTCTTTCAGGCCGCGTCTTAATACTG-3′ and 5′-CAGCGAGTCCTGGTGCCGAGAGTTGCCTGTTGTC-3′ for the Cys⁶⁷ to Ala⁶⁷ mutation and 5′-GGTACCAAGTTGGTCTTTCAGGCCGCGTCTTAATACTG-3′ and 5′-CTGAGGAAACAAACGTGGTACCTTCTAGAAGC-3′ for the Cys¹⁰₈ to Ala¹⁰₈ mutation. The mutations were confirmed by sequencing.

**Evaluation of Arsenic Resistance and Response to Oxidoreductaging Chemicals**—*E. coli* strains with the vector pTriplEx2 or the recombinant pTriplEx-Pv5–6 or their mutated versions or pTriplEx-EcGrx3 were cultured in half-strength LB medium with 20 mM MES-KOH, pH 6.5, and 0.1 mM isopropyl β-D-thiogalactoside (IPTG), containing different levels of sodium arsenate or sodium arsenite and 50 mg liter⁻¹ carbencillin. The cultures, inoculated with late log phase cells, were incubated at 37 °C, 200 rpm, and culture turbidity was measured after 18 h. Similar assays and conditions were used when *E. coli* were tested for tolerance to hydrogen peroxide, methyl violagen, and cadmium chloride.

**Glutaredoxin and Arsenate Reductase Assays**—Grx activity was determined with a coupled enzymatic reaction as described previously (34) by measuring the reduction of 2-hydroxyethyl disulfide (HED) in the presence of NADPH and glutathione reductase. The decrease in NADPH was monitored at 340 nm.
using a molar extinction coefficient of 6200 m⁻¹ cm⁻¹. The assay mixture contained 100 μg/ml bovine serum albumin, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, 0.1 mM Tris-HCl, 2 mM EDTA, pH 8.0, and 0.7 mM HED in a total volume of 800 μl. Non-enzymatic NADPH-dependent reduction of HED was measured for the background. In experiments evaluating arsenate induction in vitro, non-enzymatic generation of GSSG by arsenate was subtracted as a background. This background rate was less than 2% of the Grx activity.

Arsenate reductase activity was assayed using a spectrophotometric method described previously (27). The enzyme activities were expressed as μmol mg⁻¹ of protein min⁻¹.

**Immunoblot Analysis**—Polyclonal antibodies were generated, using a commercial antibody production service (GenScript, Piscataway, NJ) in rabbit against a keyhole limpet hemocyanin-conjugated synthetic peptide (named PvGrx5–50p) with the following sequence: TQHRPSWTTAQPPN. Immunoblots were done as described previously (35), using 1:10,000 dilution of the primary antibody.

**Arsenate Metabolism**—E. coli cultures were grown in half-strength LB medium with 20 mM MES-KOH, pH 6.5, and 0.1 mM IPTG, containing 2.5 mM sodium arsenate and with appropriate antibiotic for 18 h in a shaker incubator set at 37 °C and 200 rpm. Growth was monitored by measuring turbidity at 600 nm, and the cells were centrifuged. Total arsenic and arsenite were measured in the cell pellets following their extraction in 50% (v/v) methanol in water (13).

**Arsenic Speciation**—Arsenate and arsenite were separated using an arsenic speciation cartridge (Metal Soft Center, Highland Park, NJ), which retains arsenate. Total arsenic and arsenite were determined by GFAAS (Varian 240Z, Zeeman Atomic Absorption Spectrophotometer, Varian, Walnut Creek, CA) as described (36).

**RESULTS**

Phagemids excised from a P. vittata frond cDNA library (13) were introduced into E. coli XL-1 blue, and colonies were selected on LB agar containing 5 mM arsenate supplemented with carbenicillin 50 mg/liter. Clone Pv5–6 was identified based on its arsenite tolerance on the agar selection plate. pTriplEx2 vector of this construct allowed IPTG-inducible expression of the cDNA insert in all three reading frames (13).

**Pv5–6 Expression Confers Resistance to Arsenic in XL-1 Blue**—The ability of cells of E. coli strain XL-1 Blue expressing Pv5–6 to confer resistance to arsenate and arsenite was examined. Cells expressing Pv5–6 under control of the lac promoter showed significantly greater tolerance to both arsenate and arsenite when compared with cells with vector alone (Fig. 1). Resistance to arsenite was comparatively less than to arsenate (Fig. 1). In contrast, expression of Pv5–6 did not increase tolerance to hydrogen peroxide, methyl viologen, or cadmium chloride, indicating specificity for inorganic arsenic (Fig. 2). Because Pv5–6 was identified to be a glutaredoxin (see below) and it showed high overall homology only with EcGrx3 of the E. coli proteome, it was tested whether E. coli Grx3 expressed in pTriplEx2 vector will alter arsenic tolerance. E. coli Grx3 expression did not increase arsenic tolerance (Fig. 1).

**Brake Fern Glutaredoxin and Arsenic Resistance**

**FIGURE 1.** Expression of Pv5–6 in E. coli strain XL-1 Blue increased arsenate (A) and arsenite (B) tolerance. VC:XL1-Blue transformed with pTriplEx2 vector, EcGrx3:XL1-Blue refers to E. coli XL1-Blue transformed with pTriplEx-EcGrx3, and Pv5–6:XL1-Blue refers to E. coli XL1-Blue transformed with pTriplEx-Pv5–6. Values are means and standard errors for triplicate assays.

**FIGURE 2.** Expression of Pv5–6 in E. coli XL1-Blue increased resistance to arsenate (0.5 mM) and not to hydrogen peroxide (0.5 mM), methyl viologen (0.5 mM), cadmium chloride (0.5 mM) (closed bars) when compared with E. coli XL1-Blue with an empty vector (open bars). The arsenic resistance assays and resistance to oxyradical generating agents were performed as described under “Experimental Procedures.” VC:XL1-Blue refers to E. coli XL1-Blue transformed with pTriplEx vector, and Pv5–6:XL1-Blue refers to E. coli XL1-Blue transformed with pTriplEx-Pv5–6.

**Pv5–6 Is a Glutaredoxin Homologue**—The Pv5–6 cDNA (785 bp) encodes a 184-residue polypeptide whose sequence showed 66% identity and 78% homology with AtGrxcp, earlier reported as cation exchanger (CAX)-interacting protein (CXIP) of Arabidopsis (22, 37), a member of the protein kinase C-interacting cousin of thioredoxin homology domain
Brake Fern Glutaredoxin and Arsenic Resistance

AtGRXcp MALRSVKTPF-LITFVAVSSVTNKPSH1RF3LPTLSALVHNQLSFLGSHN----LKL 55
PvGRX5 MASRAVQTVGVLGGSQGDSLLHSSTSTNSQFLLLHFSPTFILLSKRATVLISSQHRPSTTAQ 60
ScGLRX5 -----------------------------MFLKPN1RISFSP1LRAKTLRYQR--- 27
EcGRX3

AtGRXcp KPTKFCBASS--------ALTQPDKDTELEKVXSKVLMKMGTRDFPPGCFNPTTVQIQLN 109
PvGRX5 FFPYR1CNSGQVAPTLSVELKKEAVDVKFYTVSKVLPMKGLFPCGSGVQVQLNS 120
ScGLRX5 -----------------------------WLSFIEHKAIDEASAPVP1LPMGTFFPPPGRFFHATVICTGQLGN 72
EcGRX3 -----------------------------MANVEITYFET-----CPYCHRKAALLSS 24

AtGRXcp VKEKAMS----------------- 173
PvGRX5 IEKAMS----------------- 184
ScGLRX5 LEEAGAVVPEEETKDR 150
EcGRX3 LK----------------------------- 83

FIGURE 3. Alignment of deduced amino acid sequence of Pv5–6 (PvGrx5) with that of Arabidopsis GRXcp sequence (accession number AT157988), Saccharomyces cerevisiae ScGLRX5 (accession number Q92784) and E. coli EcGRX3 (accession number NP_290193.1). Potential Grx motifs in Pv5–6 CRSS and CGFS are shown by underlines and bold, and conserved identical amino acid residues are shown by asterisks.

The recombinant Pv5–6 protein (rPv5–6) catalyzed thioldisulfide oxidoreduction as assayed by reduction of HED by GSH (Fig. 5A). The pH optimum was 8, and the $k_m$ (max) and $V_{max}$ (mM/min$^{-1}$-mg$^{-1}$ protein) values were 1.06 ± 0.6 and 54 ± 8 for HED, and 2.2 ± 0.4 and 68 ± 6 for GSH, respectively. Based on this Grx activity, the protein encoded by Pv5–6 cDNA was named PvGRX5.

To examine the effect of arsenic on rPvGRX5 activity, 10 mM sodium arsenate or sodium arsenite was added before HED. A 1.6-fold increase in Grx activity was observed with the addition of arsenate (Fig. 5B) but not by arsenite (not shown). Induction by arsenate was not observed in assays where purified E. coli Grx2 was used (Fig. 5B).

rPvGRX5 did not exhibit arsenate reductase activity in vitro as measured using a spectrophotometric method (data not shown). In an arsenate reductase assay of E. coli arsenite reduc-
tase, rPvGRX5 replaced E. coli Grx, but about 10-fold less effec
tively than E. coli Grx2 (data not shown).

Grx Activity of PvGRX5 Was Linked to Cellular Arsenic Resistance—PvGRX5 had two potential motifs that may be important for Grx activity and arsenic tolerance: 67CXXS$^{70}$ and 108CGFS$^{111}$. In E. coli, only the first cysteine of the CXXS motif is required for arsenate resistance, and the second cysteine can be changed to serine without effect on arsenate resistance (24). When Cys$^{67}$ of PvGRX5 was mutated to an alanine residue, cellular arsenate resistance was lost (Fig. 6). Specific activities of Grx in Pv5–6/6A67::XL1-BL was comparable with vector control and was only 20% of Grx activity found in Pv5–6::XL1-BL (Fig. 6, inset), suggesting loss of Grx activity due to the mutation. When Cys$^{108}$ was mutated to an alanine residue, there was partial loss of Grx activity and arsenic tolerance (data not shown).

The Role of PvGRX5 in Arsenic Resistance Is Independent of ars Operon Genes—To examine whether PvGRX5 depended on the expression of the ars operon genes of E. coli to increase cellular arsenic resistance, Pv5–6 was introduced into AW3110 (Δars) (26) and WC3110 (ΔarsC) (27) to evaluate their arsenic tolerances. Expression of the PvGRX5 signifi-
significantly increased the resistance of AW3110 and WC3110 to arsenate and arsenite when compared with vector-alone controls in these strains (Fig. 7, A and B). These increases in resistance were not greater than that found in the wild-type strain W3110 (Fig. 7, A and B).

**FIGURE 5.** Recombinant PvGrx5 is a functional glutaredoxin and is activated by arsenate. A, purified recombinant PvGRX5 was assayed in a classical Grx assay with (filled circle) and without (empty circle) the substrate HED and without protein (triangle). B, Grx-specific activity in assays with HED substrate for rPvGRX5 or EcGrx2 with or without 10 mM sodium arsenate in the assay. (See “Experimental Procedures” for details.)

**FIGURE 6.** Cys67 is essential for arsenite tolerance and Grx activity. Arsenite tolerance of E. coli XL1-Blue, XL-1 Blue transformed with Pv5–6 (black squares), or Pv5–6 A67 mutant is shown. Inset, Grx-specific activity (µmol·mg⁻¹·min⁻¹) of crude protein extracts from E. coli XL1-Blue transformed with Pv5–6 A67 (A), vector control (B) and Pv5–6 (C). Values are means and standard errors for three determinations. Pv5–6A67:XL1-BL refers to E. coli XL1-Blue transformed with pTriplEx-Pv5–6 A67 vector, VC:XL1-BL refers to E. coli XL1-Blue transformed with pTriplEx2 vector, and Pv5–6:XL1-BL refers to E. coli XL1-Blue transformed with pTriplEx-Pv5–6.

**FIGURE 7.** PvGRX5 functions independently of ars operon but depends on GlpF. A and B, response to arsenate (A) and arsenite (B) to AW3110 transformed with vector control (open circles) or with Pv5–6 (closed circles), WC3110 transformed with vector control (open triangles) or Pv5–6 (closed triangles) and parental strain W3110 (closed squares). VC:AW3110 refers to E. coli AW3110 transformed with pTriplEx2 vector, VC:XL1-BL refers to E. coli XL1-Blue transformed with pTriplEx2, and Pv5–6:XL1-BL refers to E. coli XL1-Blue transformed with pTriplEx-Pv5–6. C, response to arsenate to OSBR-1 (open circles) and OSBR-1 transformed with Pv5–6 (closed circles). Values are means and standard errors for three determinations. Pv5–6:OSBR1 refers to E. coli OSBR1 transformed with pTriplEx-Pv5–6.

The Expression of PvGRX5 in an Aquaglyceroporin Deletion Mutant Does Not Alter Its Arsenic Tolerance—To examine whether PvGRX5 required the aquaglyceroporin for its role in arsenic tolerance, Pv5–6 was introduced into strain OSBR1, in which the glpF gene for the aquaglyceroporin responsible for arsenite uptake was disrupted in addition to deletion of the ars
recombinant PvGRX5 protein and showed it to be a 20-kDa protein, expected for the enterokinase-digested recombinant protein (20.266 kDa). Specific recognition by polyclonal antibodies against the peptide (Thr<sup>50</sup> to Asn<sup>63</sup>) is consistent with the recombinant protein being full-length. We demonstrated that PvGRX5 is a functional glutaredoxin by a combination of functional expression in <i>E. coli</i> and biochemical characterization of purified recombinant PvGRX5 (Fig. 5A). Consistent with a role in arsenic metabolism, <i>P. vittata</i> Grx was activated about 2-fold by arsenate (Fig. 5B).

Because Grxs are involved in oxidative stress tolerance (22, 41), we examined the ability of PvGrx5 to confer resistance to inhibitory concentrations of hydrogen peroxide, cadmium chloride, and methyl viologen. Cells of <i>E. coli</i> expressing PvGrx5 were more resistant to arsenate and arsenite (Fig. 1) when compared with controls but not with the oxidants tested (Fig. 2), suggesting that PvGrx5 specifically functions in arsenic tolerance. PvGrx5 was unique in increasing cellular arsenic resistance since <i>E. coli</i> Grx3 expression in the same vector did not increase arsenic resistance (Fig. 1), suggesting that the phenotype observed here is only due to the expression of PvGrx5.

PvGRX5 contained two Grx catalytic motifs: <sup>97</sup>CXXS<sup>100</sup> and <sup>108</sup>C9FS<sup>111</sup>, characteristic of subgroup I and subgroup II of plant Grxs, respectively (23). Mutagenesis of Cys<sup>67</sup> resulted in loss of both Grx activity and arsenic resistance (Fig. 6), showing that arsenic resistance is dependent on the presence of Cys<sup>67</sup> of the CXXS motif.

In <i>E. coli</i>, the chromosomai <i>ars</i>RBC operon confers moderate resistance to arsenite. ArsR is an arsenic-responsive transcriptional regulator, ArsC is an arsenite reductase that reduces As(V) to As(III), and ArsB is an arsenite extrusion protein (42). Grx2 in <i>E. coli</i> is the most effective hydrogen donor for ArsC (24). Therefore, we tested whether the role of PvGRX5 in arsenic tolerance was due to its interaction with one or many <i>ars</i> operon gene products in <i>E. coli</i>. When PvGRX5 was expressed in WC3110, an arsenate reductase minus strain (26), and AW3110, a strain with a deletion for the entire <i>ars</i> operon (28), OSBR1 with and without Pv5–6 had comparable resistance to arsenate (Fig. 7C) and arsenite (data not shown).

Expression of PvGRX5 Decreases Intracellular Arsenic—The intracellular amounts of As(III) and As(V) in cells of AW3110 and OSBR1 expressing PvGRX5 or vector control were determined following exposure of cells to 2.5 mM as(V) for 18 h (Fig. 8). Both these strains transformed with vector control contained arsenate and arsenite at comparable levels (Fig. 8). AW3110 cells expressing Pv5–6 had 3-fold less As(III) than the vector control (Fig. 8). In contrast, there was no significant change in the cellular As(V) content because of the expression of Pv5–6 (Fig. 8). Similar results were obtained when Pv5–6-expressing XL1-BL cells were analyzed (data not shown). In contrast, in OSBR1, arsenate and arsenite levels were not significantly different when vector or Pv5–6 was expressed (Fig. 8).

**DISCUSSION**

We identified a <i>P. vittata</i> frond cDNA that, upon expression, increased bacterial resistance to inhibitory levels of both arsenate and arsenite (Fig. 1). Characterization of this cDNA revealed a new plant Grx that appears to have a role in regulating cellular arsenite levels. This validates our sequence homology-independent, high throughput cDNA cloning strategy (13). Given the current lack of mutants, genome sequences, and transformation methods in this extraordinary fern (39), functional cloning methods appear to be superior for understanding genes involved in arsenic resistance and hyperaccumulation in <i>P. vittata</i> (12, 13).

The cDNA Pv5–6 encodes a protein homologous to AtGRXcp, <i>Arabidopsis thaliana</i> glutaredoxin with a PICOT domain (Fig. 3) (22). AtGRXcp was initially identified in a screen for PICOT domain-containing proteins able to activate <i>Arabidopsis</i> CAX1 protein involved in Ca<sup>2+</sup>/H<sup>+</sup> antiport activity in a yeast expression system (37). <i>Arabidopsis</i> CAX proteins have a role in ion homeostasis (40). Recent functional analyses of AtGRXcp indicated that this protein is involved in protecting proteins from oxidative damage (22). Although the biochemical function of AtGRXcp was inferred to be a glutaredoxin, this was not demonstrated (22). In this study, we have overexpressed a...
situation in Sinorhizobium meliloti, which lacks an active efflux system for arsenite and requires an aquaglyceroporin for arsenate resistance (44).

Arsenic speciation in cellular extracts showed that E. coli AW3110 and OSBR1 had both arsenate and arsenite following exposure to 2.5 mM sodium arsenate in the medium for 18 h, under the conditions of the experiment (Fig. 8). Although the nature of arsenate reduction in these strains lacking the ars operon is not understood at present, cells of E. coli AW3110 expressing PvGrx5 had significantly lower intracellular levels of arsenite than vector controls (Fig. 8), presumably because cells with PvGrx5 reduce arsenate to arsenite so much faster than cells lacking an arsenate reductase and/or efflux As(III) much faster than cells without the fern Grx.

Grxs catalyze reversible deglutathionylation of protein-S-S-glutathione-mixed disulfides. Glutathionylation of proteins have been ascribed both metabolic and regulatory importance in keeping the redox homeostasis of cells (46). Our studies suggest for the first time that PvGrx5 directly or indirectly interacts with a protein involved in arsenic transport homologous to bacterial glpF, a transmembrane protein (28, 44). Plants contain a family of aquaglyceroporins, including proteins with high homology to those implicated in arsenite transport in other organisms (47). This implies that PvGrx5 in P. vittata frond might possibly regulate a vacuolar glpF homolog (e.g. a tonoplast intrinsic protein) to alter arsenite transport into the vacuole.

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