The role of phytochelatins in arsenic tolerance in the hyperaccumulator *Pteris vittata*

F. J. Zhao[1], J. R. Wang[1], J. H. A. Barker[2], H. Schat[3], P. M. Bleeker[3] and S. P. McGrath[1]

[1]Agriculture and Environment Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK; [2]Crop Performance and Improvement Division, Long Ashton Research Station, Long Ashton, Bristol, BS41 9AF, UK; and [3]Department of Ecology and Physiology of Plants, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

Author for correspondence:
F. J. Zhao
Tel: +44 1582763133
Fax: +44 1582760981
Email: Fangjie.Zhao@bbsrc.ac.uk;

Received: *9 January 2003*
Accepted: *11 March 2003*

doi: 10.1046/j.1469-8137.2003.00784.x

Summary

• *Pteris vittata* was the first identified arsenic (As) hyperaccumulator. Here we investigated whether phytochelatins (PCs) are involved in the hypertolerance of arsenic by *P. vittata*.

• *P. vittata* was exposed to 0–500 μM arsenate for 5 d, or to 50 μM arsenate for 0–7 d. In addition, L-buthionine-sulphoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, was used in combination with different arsenate exposures. The relationships between As accumulation and the concentrations of PCs and glutathione (GSH) were examined.

• PC synthesis was induced upon exposure to arsenate in *P. vittata*, with only PC_2 detected in the plant. The As concentration correlated significantly with PC_2 concentration in both roots and shoots, but not with GSH. The molar ratio of PC-SH to As was *c.* 0.09 and 0.03 for shoots and roots, respectively, suggesting that only a small proportion (1–3%) of the As in *P. vittata* can be complexed with PCs. In the presence of arsenate, addition of BSO decreased PC_2 concentrations in roots and shoots by 89–96% and 30–33%, respectively. BSO alone was found to inhibit root growth of *P. vittata* markedly.

• The results suggest that PCs play a limited role in the hypertolerance of As in *P. vittata*.

Key words: *Pteris vittata*, arsenic (As), hyperaccumulation, phytochelatins, tolerance.

Introduction

The brake fern *Pteris vittata* is the first identified arsenic (As) hyperaccumulator (Ma *et al.*, 2001). It can accumulate > 20 000 mg As kg^{-1} in the shoot (frond) dry weight (d. wt). Furthermore, the bioconcentration factor, defined as the ratio of shoot As concentration to soil As concentration, was greater than 10. *P. vittata* is also hypertolerant to As, with a toxicity threshold value of about 10 000 mg As kg^{-1} d. wt in shoots (Tu & Ma, 2002; Wang *et al.*, 2002). This compares with threshold values of 10–100 mg As kg^{-1} d. wt for As nontolerant plants (Kabata-Pendias & Pendias, 1992). More recently, several other fern species in the order Pteridales have been identified to be As hyperaccumulators (Visoottiviseth *et al.*, 2002; Zhao *et al.*, 2002). These plants may have potential for phytoremediation of As-contaminated soils or waters. Moreover, these plants offer an interesting model for studying As metabolism in plants.

Inorganic As species are generally highly phytotoxic. Arsenate is a phosphate analogue and can disrupt phosphate metabolism, whereas arsenite reacts with sulfydryl groups of enzymes and tissue proteins, leading to inhibition of cellular function and death (Meharg & Hartley-Whitaker, 2002). In As nonhyperaccumulating plant species, two mechanisms of As tolerance have been identified. First, tolerant plants have a decreased uptake of arsenate due to a suppression of the high-affinity phosphate uptake system (Meharg & Macnair, 1991, 1992; Meharg & Hartley-Whitaker, 2002). Second, arsenate

taken up by plants is detoxified through reduction to arsenite, which is subsequently complexed with thiols, particularly phytochelatins (PCs). There are several lines of evidence for an important role of PCs in As tolerance. First, exposure to As induces a large response in the synthesis and accumulation of PCs in a number of plant species (Grill *et al.*, 1987; Maitani *et al.*, 1996; Sneller *et al.*, 1999; Schmöger *et al.*, 2000). Second, As-tolerant populations of *Holcus lanatus* were found to accumulate more PCs than nontolerant ones under the same degree of As stress (Hartley-Whitaker *et al.*, 2001; Hartley-Whitaker *et al.*, 2002). Third, inhibition of PC synthesis by a treatment with L-buthionine-sulphoxime (BSO), a potent inhibitor of γ-glutamylcysteine synthetase, leads to hypersensitivity to As in both As nontolerant and tolerant plants (Schmöger *et al.*, 2000; Hartley-Whitaker *et al.*, 2002; Schat *et al.*, 2002). Fourth, an *Arabidopsis thaliana* mutant lacking functional PC synthase was significantly more sensitive to As toxicity than the wild type (Ha *et al.*, 1999). Fifth, X-ray absorption spectroscopy reveals that the majority of As in the leaves of *Brassica juncea* and *A. thaliana* was coordinated to thiols (Pickering *et al.*, 2000; Dhankher *et al.*, 2002). Furthermore, intact PCs-As complexes have been isolated from plant tissues (Sneller *et al.*, 1999; Schmöger *et al.*, 2000).

The tolerance to As in *P. vittata* is at least an order of magnitude greater than in As nonhyperaccumulating plants. The mechanisms of As hypertolerance in *P. vittata* have not been elucidated. Arsenic extracted from the shoots of *P. vittata* appeared to be present mainly as inorganic arsenite (Francesconi *et al.*, 2002; Wang *et al.*, 2002). However, there is a possibility that the extraction procedure and subsequent speciation analysis may not preserve thiol-As complexes. Thus, the objective of this study was to investigate whether PCs are involved in the hypertolerance to As in the hyperaccumulator *P. vittata*.

Materials and Methods

Plant culture

Spores of *P. vittata* L. (produced from adult plants of the Florida accession, kindly provided initially by Dr Lena Ma, University of Florida, Gainesville, FL, USA) were germinated on moist potting compost. At the two-frond stage, sporelings were transplanted individually into 8-cm pots containing a general-purpose potting compost, and allowed to grow to the three–four frond stage. Plant roots were then washed carefully with tap water to remove adhering compost and transferred to 250-ml pots (one plant per pot) containing a nutrient solution, which was a modified Hoagland solution with $^1/_2$ strength major nutrients and full strength micronutrients (except that Fe was supplied as Fe-EDTA at 100 μM) (Hewitt, 1966). Solution pH was buffered at around 6.0 using 5 mM MES (2-morpholino-ethanesulphonic acid). The nutrient solution was aerated continuously and renewed every 5 d. Plants were grown in the hydroponic culture for 3 wk before experiments started. The hydroponic culture and all experiments were conducted inside a controlled environment growth chamber with the following conditions: 16 h light period with a light intensity of 350 μmol m^{-2} s^{-1}, 25°C/20°C day/night temperatures and 60% relative humidity.

Experiment 1

P. vittata plants were exposed to increasing concentrations of arsenate (0, 10, 50, 100 and 500 μM) for 5 d. Arsenate was supplied as Na_2HAsO_4, and the composition of nutrients was the same as in the preculture. Each treatment was replicated three-fold. After 5 d of exposure, plant roots and shoots were rinsed thoroughly with deionized water, blotted dry and separated. Roots and shoots were frozen in liquid nitrogen and freeze-dried at −60°C for 3 d. Samples were ground and stored in vials at −80°C until analysis.

Experiment 2

P. vittata plants were exposed to 50 μM arsenate (Na_2HAsO_4) for 0, 1, 3 and 7 d. This concentration was chosen because it was not toxic to *P. vittata* in preliminary experiments. The composition of nutrients was the same as in the preculture. On each sampling occasion, plants from three replicate pots were harvested and treated as described above.

Experiment 3

This experiment was conducted to investigate the effect of L-buthionine-sulphoximine (BSO) and arsenate exposure on root growth and PC synthesis. After 3 wk of preculture under hydroponic conditions, plants in 18 pots were treated with 500 μM BSO for 5 d. Another group of 18 plants was not treated with BSO. Thereafter, plants were exposed to 0, 500 and 1000 μM arsenate (Na_2HAsO_4), with or without 500 μM BSO, for a further 5 d. Each As and BSO treatment was replicated in six pots. Root elongation after 5 d exposure was measured in three pots of each treatment using the charcoal staining method (Schat & Ten Bookum, 1992). The remaining three pots for each treatment, unstained with charcoal, were used for the analysis of As and PCs. The plants were harvested and treated as described for Experiment 1.

Analysis

Ground plant materials were digested with a mixture of HNO_3/$HClO_4$ (85/15, v/v) and the concentrations of As were determined using inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Fisons-ARL Accuris, Ecublens, Switzerland). Blanks and internal standards were included for quality assurance.

Freeze-dried plant material (20 mg) from Experiment 3 was extracted with 5 ml 30 mM ammonium phosphate buffer at

pH 5.6 in a sonication bath for 30 min. The suspension was centrifuged and filtered through a Whatman No. 42 filter paper. As(III) and As(V) were determined in the extracts using automated hydride-generation atomic absorption spectrometry (Glaubig & Goldberg, 1988).

Extraction and analysis of PCs and glutathione (GSH) were performed using a slightly modified method described by Sneller *et al.* (2000). Freeze-dried plant material (10 mg) was ground in a mortar and pestle in 2 ml 6.3 mM DTPA (diethylenetriaminepentaacetic acid) with 0.1% (v/v) trifluoroacetic acid at 4°C. *N*-acetyl cysteine was added during grinding as an internal standard. The contents were centrifuged at 13 000 ***g*** for 10 min at 4°C and the supernatant filtered through a Costar Spin-X centrifuge tube with a 0.22-µm nylon filter. The thiols in the extract (250 µl) were derivatized with 10 µl 25 mM monobromobimane, together with 450 µl 200 mM 4-(2-hydroxyethyl)-piperazine-1-propanesulphonic acid buffer at pH 8.2 and 6.3 mM DTPA. Derivatization was carried out at 45°C for 30 min. The reaction was stopped by the addition of 300 µl of 1 M methanesulphonic acid. GSH and PCs were separated on two tandemly arranged Nova-Pak C_{18} columns (6 nm, 4 µm, 3.9 × 150 mm, Waters, Milford, MA, USA) at 37°C, using a slightly concave gradient of 12% to 25% (v/v) methanol for 15 min and then a linear gradient from 25% to 50% (v/v) methanol from 15 to 40 min. Fluorescence was monitored using a Waters 474 fluorescence detector. HPLC peaks were identified by mixing some of the *P. vittata* samples with a *Silene vulgaris* sample, which has a known PC composition established previously through amino acid analysis of the purified peak fraction. Reduced GSH was used as an external standard. PC concentrations were calculated as µmol GSH equivalent g^{-1} d. wt and corrected for derivatization efficiency (Sneller *et al.*, 2000).

Analysis of variance was performed on all data. Least significant difference was used to compare treatment means.

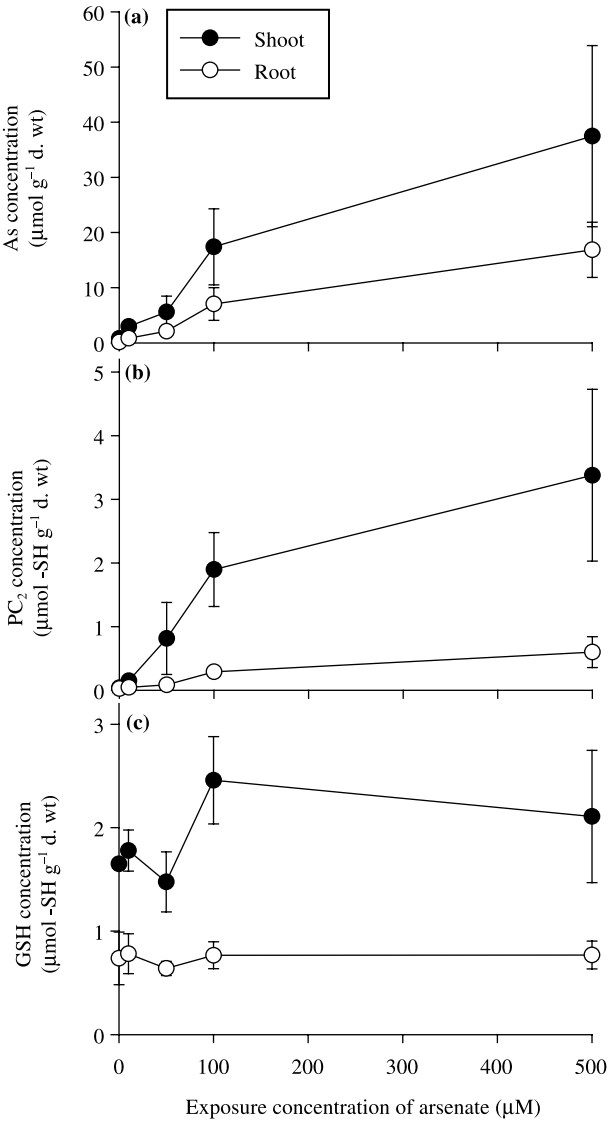

Fig. 1 Effects of arsenate exposure for 5 d on the concentrations of As (a), PCs (b) and GSH (c) in *Pteris vittata*. Bars represent ± SEs.

Results

Experiment 1: effects of arsenate exposure concentration

There were no signs of phytotoxicity in *P. vittata* after 5 d exposure to arsenate up to 500 µM. The concentrations of As in both roots and shoots increased significantly ($P < 0.05$) with increasing As concentration in the nutrient solution (Fig. 1a). In the 100 and 500 µM As treatments, the concentration of As in the shoots exceeded 1000 µg g^{-1} d. wt (13.3 µmol g^{-1}), which is the threshold value used to define As hyperaccumulation. Furthermore, the concentrations of As in the shoots were greater than those in the roots, indicating an efficient root to shoot translocation.

In the control treatment (no As), the concentrations of PCs in both roots and shoots were negligible. Arsenate exposure induced PC synthesis in both roots and shoots of *P. vittata* ($P < 0.05$; Fig. 1b). Only PC_2 was detected in the plants. In the HPLC chromatograph (result not shown), PC_2 in *P. vittata* coeluted exactly with the *S. vulgaris* PC_2. Since the latter is a glycine-PC (Sneller *et al.*, 2000), this result infers that the *P. vittata* PCs possess a glycine C-terminal. The concentrations of PC_2 were 3–10 fold higher in the shoots than in the roots. The concentrations of glutathione were not significantly ($P = 0.42$ and 0.97 for shoot and root GSH, respectively) influenced by the As treatments (Fig. 1c).

Experiment 2: effects of arsenate exposure time

In this experiment, *P. vittata* was exposed to 50 µM arsenate for up to 7 d. Arsenic accumulation by *P. vittata* increased with exposure time (Fig. 2a). By day 3, the concentration of As in the shoots was more than double that in the roots. The

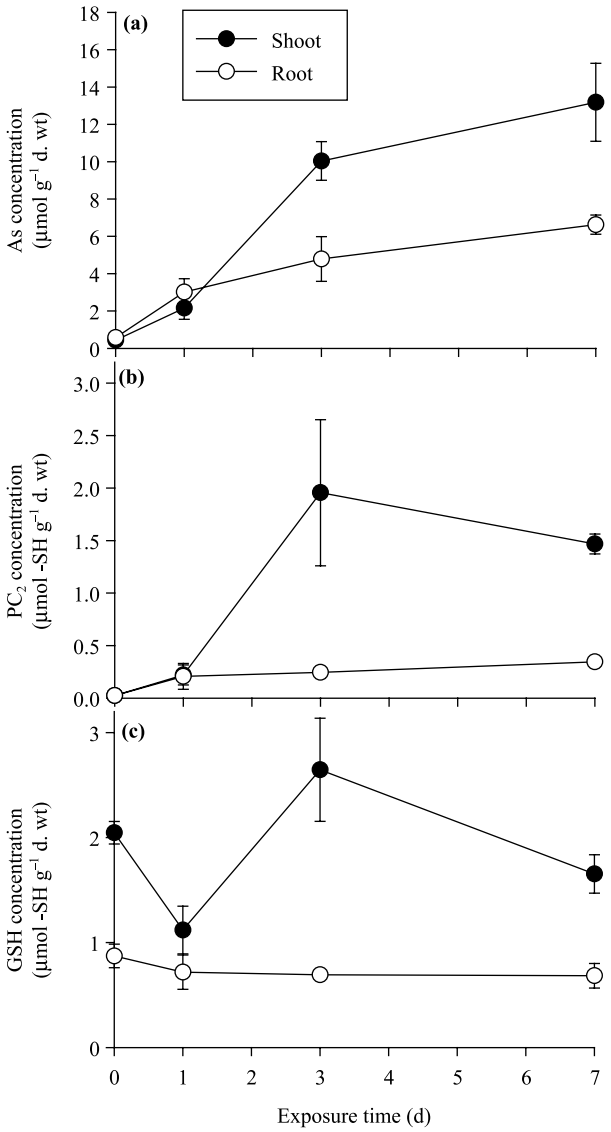

Fig. 2 Effects of arsenate exposure time on the concentrations of As (a), PCs (b) and GSH (c) in *Pteris vittata*. Bars represent ± SEs.

synthesis of PC_2 was induced by As exposure (Fig. 2b). In the shoots, PC_2 concentration appeared to peak on day 3 and remained at a similar concentration on day 7. The concentration of PC_2 in the roots was much smaller than that in the shoots. The concentration of GSH in the roots was not significantly ($P = 0.64$) influenced by the duration of As exposure (Fig. 2c). Shoot GSH level appeared to decrease during the first day of arsenate exposure and then recovered by day 3 ($P = 0.048$).

Fig. 3 shows the relationships between As, PC and GSH concentrations in roots and shoots of *P. vittata*. The data represent individual replicates for all treatments in Experiments 1 and 2. In both roots and shoots, PC concentration correlated positively and significantly ($P < 0.001$) with As concentration (Fig. 3a). The slopes, which represent the molar ratio of the PC-SH to As in the plants, were 0.09 and 0.03 for

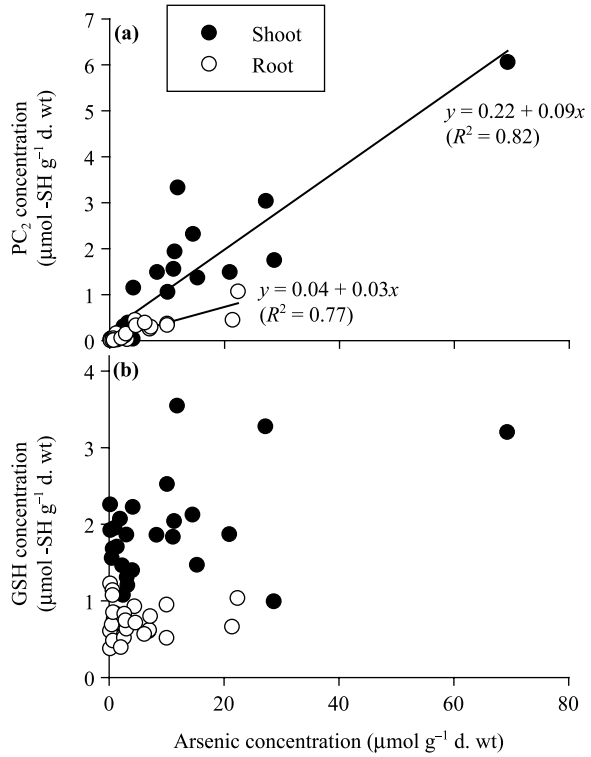

Fig. 3 Relationships between the concentrations of As and PCs (a) and GSH (b) in *Pteris vittata*. Data are from Experiments 1 and 2.

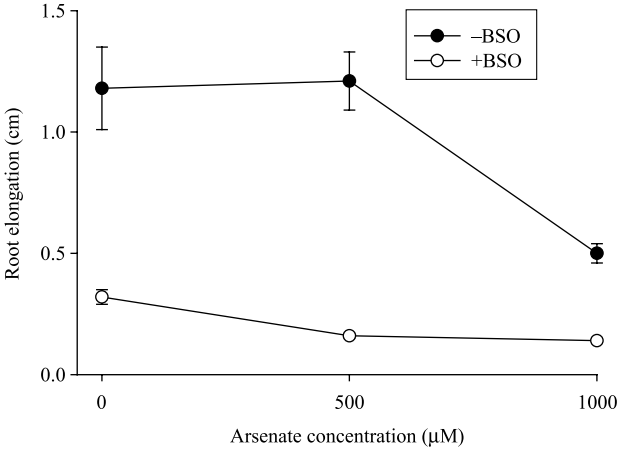

Fig. 4 Effects of L-buthionine-sulphoximine (BSO) and arsenate exposure on root elongation in *Pteris vittata*. Bars represent ± SEs.

the shoot and root tissues, respectively. By contrast to PC, there was no significant correlation between GSH and As concentrations (Fig. 3b).

Experiment 3: effects of BSO

Without BSO in the nutrient solution, root elongation during the 5-d treatment period was not significantly affected by 500 μM arsenate, but was inhibited by 58% when 1000 μM arsenate was present (Fig. 4). Addition of 500 μM

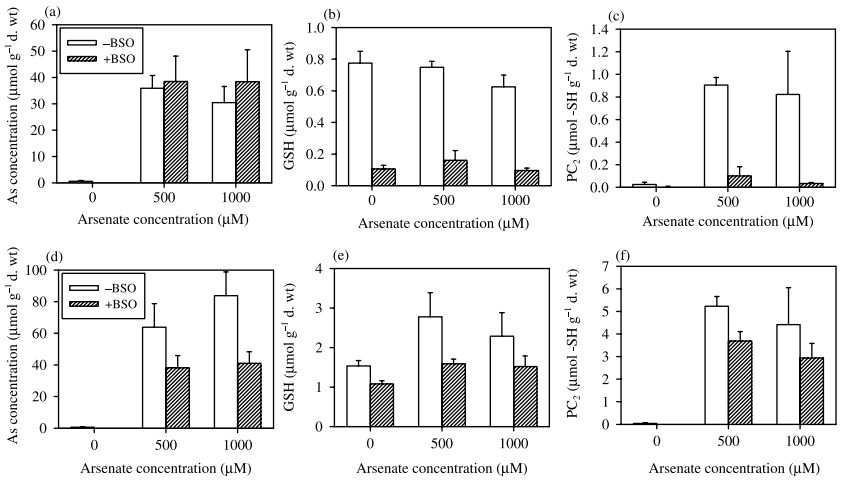

Fig. 5 Effects of arsenate and L-buthionine-sulphoximine (BSO) treatments on the concentrations of As, GSH and PCs in the roots (a–c) and shoots (d–f) of *Pteris vittata*. Bars represent ± SEs.

BSO inhibited root elongation by 73% in the absence of arsenate. In the presence of BSO, addition of 500 μM arsenate decreased root elongation by 50%, suggesting the possibility of an increased sensitivity of *P. vittata* to As toxicity. However, the severe effect of BSO on root elongation masked the effect of arsenate exposure. Phytotoxicity symptoms appeared in the shoots in the 1000 μM arsenate treatment, but the degree of phytotoxicity appeared similar with or without BSO.

Fig. 5 shows the concentrations of As, GSH and PC_2 in the roots and shoots of *P. vittata* in Experiment 3. The BSO treatment decreased shoot As concentration significantly ($P < 0.05$; Fig. 5d), but had no significant effect on root As concentration (Fig. 5a). The effect of BSO on the As accumulation in shoots may be a result of the inhibitory effect of BSO on root growth. Addition of BSO decreased the concentrations of GSH in the roots by 83% ($P < 0.001$, Fig. 5b). The BSO treatment also decreased the concentration of PC_2 in the roots by 89–96% in the + As treatments ($P < 0.001$; Fig. 5c). In comparison, the effects of BSO on the concentrations of GSH and PC_2 in the shoots were much smaller (30–43% reduction; $P < 0.05$ and $P = 0.13$ for GSH and PC_2, respectively; Fig. 5e,f).

The root and shoot tissues from the As-exposed treatments were extracted with ammonium phosphate buffer at pH 5.7 for the determination of As speciation. This extraction procedure was found to extract on average 98% of the total As. In the shoot extracts, As(III) accounted for 85–90% of the total As, with the remaining proportion as As(V) (Table 1). In the roots, As(V) was the predominant species, accounting for 81–97% of the total As in the extracts, whilst As(III) represented only 3–19%. The BSO treatment decreased the proportion of As in the roots present as As(III) significantly ($P < 0.001$), but had no significant effect on As speciation in the shoots.

Discussion

The results from all three experiments confirm the extraordinary ability of *P. vittata* to take up and transport As to the shoots. Wang *et al.* (2002) showed that arsenate is taken up via the phosphate uptake systems in *P. vittata*, similarly to As nonaccumulating plants (Asher & Reay, 1979; Ullrich-Eberius *et al.*, 1989; Meharg & Hartley-Whitaker, 2002). Clearly, *P. vittata* does not employ the As resistance mechanism commonly found in many As tolerant plants, that is a decreased arsenate uptake through suppressed high affinity phosphate transporters (Meharg & Macnair, 1992; Meharg & Hartley-Whitaker, 2002). In agreement with previous studies (Ma *et al.*, 2001; Francesconi *et al.*, 2002;

Table 1 Arsenic speciation in the shoots and roots of *Pteris vittata* from Experiment 3

		Shoot			Root		
As treatment (μM)	BSO treatment (μM)	Total As (μmol g^{-1} d. wt)*	% as As(III)	% as As(V)	Total As (μmol g^{-1} d. wt)*	% as As(III)	% as As(V)
500	0	69.1 ± 14.5	84.7 ± 2.5	15.3 ± 2.5	36.8 ± 4.9	13.0 ± 2.0	87.0 ± 2.0
	500	40.3 ± 8.8	89.7 ± 1.0	10.3 ± 1.0	32.8 ± 7.4	4.2 ± 0.7	95.8 ± 0.7
1000	0	71.6 ± 16.9	89.9 ± 3.3	10.1 ± 3.3	34.2 ± 6.1	18.6 ± 3.4	81.4 ± 3.4
	500	40.0 ± 5.1	90.5 ± 2.7	9.5 ± 2.7	39.1 ± 12.0	3.2 ± 1.4	96.8 ± 1.4

*Total As concentration extracted with ammonium phosphate buffer and determined by hydride-generation atomic absorption spectrometry (HG-AAS). Values are means ± SEs. BSO, L-buthionine-sulphoximine.

Wang *et al.*, 2002), we found that As(III) was the predominant species of As in the shoots of *P. vittata*, whereas in the roots As was present mainly as As(V). Whether As(III) was present as aqueous arsenite species or as complexed arsenite cannot be determined by the speciation method used in this study. However, evidence from studies using more sophisticated methodologies, for example HPLC-ICP-MS and X-ray absorption spectroscopy, indicates that As(III) in the leaves of *P. vittata* is present primarily as aqueous arsenite species (Wang *et al.*, 2002; Webb *et al.*, 2003). The difference between roots and shoots in As speciation suggests that leaves may be the main location of arsenate reduction. Reduction of arsenate to arsenite is mediated by the arsenate reductase enzyme in a glutathione-dependent reaction in both *Escherichia coli* (Gladysheva *et al.*, 1994) and *Saccharomyces cerevisiae* (Mukhopadhyay *et al.*, 2000). A similar mechanism may operate in *P. vittata*, although this has not been established. Higher concentrations of GSH were found in the shoots of *P. vittata* than in the roots (Figs 1, 2, 3 and 5), which would favour arsenate reduction in the leaves. The reduction of arsenate to arsenite in roots appeared to be inhibited by the BSO treatment (Table 1), probably as a result of a marked decrease in the concentration of GSH in the BSO-treated roots.

Arsenite has a high affinity to thiols and complexation of arsenite by PCs may be expected. Exposure to As has been shown to elicit synthesis and accumulation of PCs in a number of plant species that are As nonhyperaccumulators (Grill *et al.*, 1987; Maitani *et al.*, 1996; Sneller *et al.*, 1999; Schmöger *et al.*, 2000; Hartley-Whitaker *et al.*, 2002; Schat *et al.*, 2002). These studies also demonstrate that PCs play an important, probably essential, role in the detoxification of arsenite in As nonhyperaccumulating plants. The results from this study show that exposure to arsenate also induced the synthesis of PC_2 in the roots and shoots of the As hyperaccumulator *P. vittata*. The concentration of PC_2 was higher in the shoots than in the roots of *P. vittata*, which may be related to a higher concentration of As in the shoots than in the roots, and the predominance of arsenite in the shoots in this plant. However, *P. vittata* differs from As nonhyperaccumulators in two aspects. First, only PC_2 was found in *P. vittata*, whereas other plant species investigated so far also contain PCs of longer chain length. Second, the concentrations of PCs in *P. vittata* determined in this study (up to 7 μmol g^{-1} d. wt) are considerably lower than the values reported for *Silene vulgaris* (Sneller *et al.*, 1999; Schat *et al.*, 2002), *Holcus lanatus* (Hartley-Whitaker *et al.*, 2001) and *Rauvolfia serpentina* cell cultures (Schmöger *et al.*, 2000), under comparable concentrations of exposure to arsenate. In Experiment 3, 1000 μM arsenate was found to inhibit root elongation by more than 50%. However, the concentrations of PC_2 in the 1000 μM arsenate treatment were similar to those in the 500 μM arsenate treatment, which did not inhibit root elongation. The results suggest that *P. vittata* has a rather limited capacity to accumulate PCs in response to As exposure.

In *R. serpentia* (Schmöger *et al.*, 2000), *S. vulgaris* (Sneller *et al.*, 1999; Schat *et al.*, 2002) and in tolerant populations of *H. lanatus* (Hartley-Whitaker *et al.*, 2002), the molar ratio of PCs-SH to As is ≥ 3 : 1. This ratio is consistent with the structural model of one arsenite being coordinated with three thiol groups, which was observed in a reconstituted As-PC_2 complex using electrospray ionization mass spectroscopy (Schmöger *et al.*, 2000). By contrast, we observed very low molar ratios of PCs-SH to As in the shoots (*c.* 0.09) and roots (*c.* 0.03) of *P. vittata*. These ratios imply that only a small proportion of the total As accumulated by the fern, *c.* 1% and 3% for roots and shoots, respectively, may be complexed with PCs, assuming a stoichiometry of three PCs-SH to one arsenite. These estimates are consistent with previous reports showing that inorganic arsenite was by far the most dominant species of As in the shoots of *P. vittata* (Francesconi *et al.*, 2002; Wang *et al.*, 2002). Furthermore, analysis using X-ray absorption spectroscopy showed that only a small proportion (*c.* 6%) of As in the leaves of *P. vittata* was coordinated to S (Webb *et al.*, 2003). By contrast, almost all of the As in the shoots of *Brassica juncea* (Pickering *et al.*, 2000) and *Arabidopsis thaliana* (Dhankher *et al.*, 2002) was found to be complexed by thiols.

Schmöger *et al.* (2000) showed that an inhibition of GSH and PC synthesis by BSO led to an increased sensitivity of *R. serpentia* cell cultures to As toxicity. Even As-tolerant populations of *H. lanatus*, *S. vulgaris* and *Agrostis castellana* were rendered hypersensitive to As toxicity when BSO was added to the nutrient solutions (Hartley-Whitaker *et al.*, 2002; Schat *et al.*, 2002). In those studies, BSO alone had no toxic effect on root growth. In the present study, however, BSO (0.5 mM) alone was found to inhibit root growth of *P. vittata* markedly. Thus, it is difficult to judge whether a markedly decreased synthesis of GSH and PC as a result of the BSO treatment led to a higher sensitivity of *P. vittata* to As toxicity. The reason for the inhibitory effect of BSO on root growth of *P. vittata* remains unclear. One possible explanation is that a sufficient level of GSH is essential for root growth of *P. vittata*. An *A. thaliana* mutant (*rml1*) that lacks the first enzyme of the GSH biosynthesis pathway exhibits defective root cell division, because GSH is required for the phase transition of the cell cycle (Vernoux *et al.*, 2000). These authors also found that the addition of 2.5 mM BSO to growth media completely inhibited root growth of wild type *A. thaliana*. Xiang *et al.* (2001) provided further evidence for a role of GSH in root growth. They showed that root growth in *A. thaliana* was decreased by 40% in the antisense plants with a low-level expression of γ-glutamylcysteine synthetase, compared with the wild type plants.

Because arsenite can be highly disruptive to metabolic processes in cytoplasm, it has to be detoxified. This may be achieved by complexation and/or vacuolar sequestration (Ghosh *et al.*, 1999). In the leaves of *P. vittata*, As appears to be localized mainly in the vacuoles of epidermal cells (Lombi

et al., 2002). Vacuolar sequestration is likely to be the main mechanism of As detoxification in this hyperaccumulator. Furthermore, the results from this study and other recent reports (Francesconi *et al.*, 2002; Lombi *et al.*, 2002; Wang *et al.*, 2002; Webb *et al.*, 2003) suggest that the bulk of the As that is sequestrated in the vacuoles is present as uncomplexed arsenite. What then is the role of PCs in the detoxification of As in *P. vittata*, considering the positive induction of PC synthesis by As exposure? It is possible that any arsenite present in the cytoplasm is complexed by PCs, thus preventing toxic effects. Cytosolic arsenite would only account for a small proportion of the total As accumulated by this species. It is not known whether PCs-As complexes, formed in the cytoplasm, are transported across tonoplast to the vacuoles. If so, PCs-As would have to dissociate in the vacuoles and PCs or their breakdown products efflux out of the vacuoles rapidly, to account for the large accumulation of As and the overall small ratios of PCs-SH to As observed. This scenario is not very likely, considering that PCs-arsenite complexes should be more stable in the vacuoles (pH *c.* 5.5) than in the cytoplasm (pH *c.* 7.5).

In conclusion, this study shows that, although arsenate exposure induces PC synthesis in *P. vittata*, the role of PCs in the detoxification of As appears to be limited. An analogy to our conclusion here is that PCs are not responsible for Cd hypertolerance in the hyperaccumulator *Thlaspi caerulescens* (Ebbs *et al.*, 2002; Schat *et al.*, 2002).

Acknowledgements

We thank Miss Sarah Dunham and Mr Adrian Crosland for technical assistance. J. Wang thanks the China Fellowship Council for a one-year fellowship. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

References

Asher CJ, Reay PF. 1979. Arsenic uptake by barley seedlings. *Australian Journal of Plant Physiology* **6**: 459–466.

Dhankher OP, Li Y, Rosen BP, Shi J, Salt D, Senecoff JF, Sashti NA, Meagher RB. 2002. Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and – glutamylcysteine synthetase expression. *Nature Biotechnology* **20**: 1140–1145.

Ebbs S, Lau I, Ahner B, Kochian L. 2002. Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescenes* (J. & C. Presl). *Planta* **214**: 635–640.

Francesconi K, Visoottiviseth P, Sridokchan W, Goessler W. 2002. Arsenic species in an arsenic hyperaccumulating fern, *Pityrogramma calomelanos*: a potential phytoremediator of arsenic-contaminated soils. *Science of the Total Environment* **284**: 27–35.

Ghosh M, Shen J, Rosen BP. 1999. Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, USA* **96**: 5001–5006.

Gladysheva TB, Oden KL, Rosen BP. 1994. Properties of the arsenate reductase of plasmid R773. *Biochemistry* **33**: 7288–7293.

Glaubig RA, Goldberg S. 1988. Determination of inorganic arsenic (III) and arsenic (III plus V) using automated hydride-generation atomic-absorption spectrometry. *Soil Science Society of America Journal* **52**: 536–537.

Grill E, Winnacker EL, Zenk MH. 1987. Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proceedings of the National Academy of Sciences, USA* **84**: 439–443.

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

Hartley-Whitaker J, Ainsworth G, Vooijs R, Ten Bookum W, Schat H, Meharg AA. 2001. Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiology* **126**: 299–306.

Hartley-Whitaker J, Woods C, Meharg AA. 2002. Is differential phytochelatin production related to decreased arsenate influx in arsenate tolerant *Holcus lanatus*? *New Phytologist* **155**: 219–225.

Hewitt EJ. 1966. *Sand and water culture methods used in the study of plant nutrition, 2nd edn.* Farnham Royal, UK: Commonwealth Agricultural Bureaux.

Kabata-Pendias A, Pendias H. 1992. *Trace elements in soils and plants, 2nd edn.* Boca Raton, FL, USA: CRC Press.

Lombi E, Zhao FJ, Fuhrmann M, Ma LQ, McGrath SP. 2002. Arsenic distribution and speciation in the fronds of the hyperaccumulator *Pteris vittata*. *New Phytologist* **156**: 195–203.

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

Maitani T, Kubota H, Sato K, Yamada T. 1996. The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. *Plant Physiology* **110**: 1145–1150.

Meharg AA, Hartley-Whitaker J. 2002. Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. *New Phytologist* **154**: 29–43.

Meharg AA, Macnair MR. 1991. The mechanisms of arsenate tolerance in *Deschampsia cespitosa* (L.) Beauv and *Agrostis capillaris* L. *New Phytologist* **119**: 291–297.

Meharg AA, Macnair MR. 1992. Suppression of the high-affinity phosphate uptake system: a mechanism of arsenate tolerance in *Holcus lanatus* L. *Journal of Experimental Botany* **43**: 519–524.

Mukhopadhyay R, Shi J, Rosen BP. 2000. Purification and characterization of Acr2p, the Saccharomyces cerevisiae arsenate reductase. *Journal of Biological Chemistry* **275**: 21149–21157.

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

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J, Bleeker PM. 2002. The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany* **53**: 2381–2392.

Schat H, Ten Bookum WM. 1992. Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68**: 219–229.

Schmöger MEV, Oven M, Grill E. 2000. Detoxification of arsenic by phytochelatins in plants. *Plant Physiology* **122**: 793–801.

Sneller FEC, van Heerwaarden LM, Koevoets PLM, Vooijs R, Schat H, Verkleij JAC. 2000. Derivatization of phytochelatins from *Silene vulgaris*, induced upon exposure to arsenate and cadmium: Comparison of derivatization with Ellman's reagent and monobromobimane. *Journal of Agricultural and Food Chemistry* **48**: 4014–4019.

Sneller FEC, Van Heerwaarden LM, Kraaijeveld-Smit FJL, Ten Bookum WM, Koevoets PLM, Schat H, Verkleij JAC. 1999. Toxicity of arsenate in *Silene vulgaris*, accumulation and degradation of arsenate-induced phytochelatins. *New Phytologist* **144**: 223–232.

Tu C, Ma LQ. 2002. Effects of arsenic concentrations and forms on arsenic uptake by the hyperaccumulator ladder brake. *Journal of Environmental Quality* **31**: 641–647.

Ullrich-Eberius CI, Sanz A, Novacky AJ. 1989. Evaluation of arsenate- and vanadate-associated changes of electrical membrane potential and phosphate transport in *Lemna gibba*-G1. *Journal of Experimental Botany* **40**: 119–128.

Vernoux T, Wilson RC, Seeley KA, Reichheld JP, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inze D, May MJ, Sung ZR. 2000. The *root meristemless 1/cadmium sensitive 2* gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. *Plant Cell* **12**: 97–109.

Visoottiviseth P, Francesconi K, Sridokchan W. 2002. The potential of Thai indigenous plant species for the phytoremediation of arsenic contaminated land. *Environmental Pollution* **118**: 453–461.

Wang J, Zhao FJ, Meharg AA, Raab A, Feldmann J, McGrath SP. 2002. Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. *Plant Physiology* **130**: 1552–1561.

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

Xiang CB, Werner BL, Christensen EM, Oliver DJ. 2001. The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. *Plant Physiology* **126**: 564–574.

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