

Arsenic Hyperaccumulation in Gametophytes of *Pteris vittata*. A New Model System for Analysis of Arsenic Hyperaccumulation¹

Luke Gumaelius, Brett Lahner, David E. Salt*, and Jo Ann Banks

Department of Botany and Plant Pathology (L.G., J.B.) and Center for Plant Environmental Stress Physiology (B.L., D.E.S.), Purdue University, West Lafayette, Indiana 47907

The sporophyte of the fern *Pteris vittata* is known to hyperaccumulate arsenic (As) in its fronds to >1% of its dry weight. Hyperaccumulation of As by plants has been identified as a valuable trait for the development of a practical phytoremediation processes for removal of this potentially toxic trace element from the environment. However, because the sporophyte of *P. vittata* is a slow growing perennial plant, with a large genome and no developed genetics tools, it is not ideal for investigations into the basic mechanisms underlying As hyperaccumulation in plants. However, like other homosporous ferns, *P. vittata* produces and releases abundant haploid spores from the parent sporophyte plant which upon germination develop as free-living, autotrophic haploid gametophyte consisting of a small (<1 mm) single-layered sheet of cells. Its small size, rapid growth rate, ease of culture, and haploid genome make the gametophyte a potentially ideal system for the application of both forward and reverse genetics for the study of As hyperaccumulation. Here we report that gametophytes of *P. vittata* hyperaccumulate As in a similar manner to that previously observed in the sporophyte. Gametophytes are able to grow normally in medium containing 20 mM arsenate and accumulate >2.5% of their dry weight as As. This contrasts with gametophytes of the related nonaccumulating fern *Ceratopteris richardii*, which die at even low (0.1 mM) As concentrations. Interestingly, gametophytes of the related As accumulator *Pityrogramma calomelanos* appear to tolerate and accumulate As to intermediate levels compared to *P. vittata* and *C. richardii*. Analysis of gametophyte populations from 40 different *P. vittata* sporophyte plants collected at different sites in Florida also revealed the existence of natural variability in As tolerance but not accumulation. Such observations should open the door to the application of new and powerful genetic tools for the dissection of the molecular mechanisms involved in As hyperaccumulation in *P. vittata* using gametophytes as an easily manipulated model system.

Arsenic (As) is a contaminant of soils and ground water in many regions of the world, including the United States (for review, see Nordstrom, 2002). Epidemiological studies conducted since the 1960s indicate that inorganic As ingested from drinking water is linked to an increased incidence of internal cancers in humans, including lung, bladder, and kidney cancers (for review, see Smith et al., 2002). The evidence of health risk from As contamination is so compelling that in 2002 the Environmental Protection Agency recommended lowering the maximum contaminant level of As from 50 to 10 $\mu\text{g}/\text{L}$, making remediation of As contaminated water an increasingly important, but expensive, concern (Smith et al., 2002).

One means to remediate As contaminated sites is by phytoremediation, i.e. using plants to remove contaminants from soils, sediments, and/or groundwater (for review, see Salt et al., 1998). In order for As phytoremediation to succeed, the phytoremediating plant must fulfill three criteria: the plant root system must be able to take up and deplete the contaminating As in

the soil; the plant must translocate and accumulate As in the shoot, which can then be harvested and processed; and the plant must have a mechanism to protect itself from the toxic effects of the As it accumulates. It has recently been shown that the fern *Pteris vittata*, a member of the Pteridaceae, meets all of these criteria (Ma et al., 2001; Chen et al., 2002; Visoottiviseth et al., 2002). Specimens of *P. vittata* obtained from a site contaminated with chromated copper arsenate were shown to tolerate and hyperaccumulate As, with up to 93% of the arsenic localized to the fronds (Ma et al., 2001; Chen et al., 2002; Visoottiviseth et al., 2002).

The physiology of As uptake and hyperaccumulation in *P. vittata* have been well characterized since its As hyperaccumulating properties were first discovered. By comparing As content in different parts of *P. vittata* sporophytes grown in the presence of As, the highest levels of As were shown to occur in fronds (Chen et al., 2002; Lombi et al., 2002; Ma et al., 2001; Tu and Ma, 2002; Tu et al., 2002) with up to 25 times more As in the fronds than in the roots (Tu and Ma, 2002). Old fronds contain as much as 13,800 mg As kg^{-1} dry biomass, an amount 142 times greater than the concentration of As in the soil in which the plants were grown (Tu et al., 2002). Within the frond, As is localized to upper and lower epidermal cells where it is likely stored in the vacuoles (Lombi et al., 2002). Fronds of *P. vittata* plants grown in the presence of

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* Corresponding author; e-mail dsalt@purdue.edu; fax 765-494-0391.

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arsenate accumulate As mostly in the As(III) oxidation state (Lombi et al., 2002; Zhang et al., 2002), while As in the root occurs predominantly as arsenate As(V) (Zhang et al., 2002). Together, these data suggest that arsenate is taken up by *P. vittata* roots, converted to arsenite shortly before or after it is rapidly translocated to the shoot, where arsenite accumulates in the epidermal cells of the fronds.

How *P. vittata* rapidly translocates As (III) from the root to the shoots, and is able to survive the exceedingly high concentrations of frond arsenite that perturbs cellular function by disrupting the sulfhydryl groups of proteins in cells (for review, see Meharg and Hartley-Whitaker, 2002), remains unknown. In some flowering plant species, arsenate, which is a phosphate analog, is thought to be taken up via phosphate transport systems (for review, see Meharg and Hartley-Whitaker, 2002). In investigating the interactions between arsenate and phosphate on arsenate uptake, Wang et al. (2002) found that the addition of phosphate to the medium in which *P. vittata* plants were grown decreased arsenate influx, while phosphate starvation increased arsenate influx. However, arsenite uptake was not affected by phosphate conditions. These results suggest that arsenate can be taken up by the *P. vittata* root system via a phosphate transporter, but do not exclude the possibility that other mechanisms of As uptake exist in this plant. Once taken up by the root, arsenate is reduced to As(III). In *Brassica juncea*, an As nonaccumulator, the majority of As(III) is conjugated in both roots and shoots by thiols (Pickering et al., 2000), most likely from glutathione or phytochelatin (Pickering et al., 2000; Schmoger et al., 2000). In *Holcus lanatus*, an arsenate-tolerant grass, detoxification of As likewise involves the formation of As-phytochelatin complexes that are thought to be sequestered in the vacuole of the cell (Meharg and Hartley-Whitaker, 2002; Raab et al., 2004). Furthermore, the Arabidopsis *cad1* mutant deficient in phytochelatin synthase is As sensitive (Ha et al., 1999). Interestingly, significant levels of thiol conjugated forms of As(III) have not been observed in *P. vittata* sporophyte (Wang et al., 2002; Zhao et al., 2003; Ze-Chun et al., 2004; Raab et al., 2004), suggesting a novel mechanism of As tolerance in *P. vittata*.

Discovering the molecular mechanisms underlying As tolerance and accumulation in the As hyperaccumulator *P. vittata* can be facilitated by identifying genes that are both necessary and sufficient for these properties. This task is difficult in *P. vittata* because the sporophyte is a slow growing perennial plant that has a large genome size of approximately 4834 Mbp (J. Banks, unpublished data) and no forward or reverse genetics tools for testing gene function. An alternative to studying As-associated traits in the sporophyte is to use the gametophyte as the target of study. *P. vittata*, like other homosporous ferns, produces and releases abundant haploid spores from the parent sporophyte plant (Fig. 1, A–C). Upon germination, each fern spore (Fig. 1, D–F) develops as a free-living, autotrophic haploid gametophyte that produces egg-forming ar-

chegonia and sperm-forming antheridia (Fig. 1, G–I). A 1-month-old *P. vittata* gametophyte consists of a tiny (<1 mm) single-layered sheet of cells, called the prothallus, with several hair-like rhizoids and a multicellular meristem that forms a notch, giving the prothallus a heart-shaped appearance (Fig. 1H). There are several advantages in using the gametophyte generation in the study of As tolerance and accumulation in *P. vittata*. First, while the sporophyte is large, morphologically complex and can take months to mature, millions of gametophytes can be cultured in liquid media quickly and uniformly under highly controlled environments, making the gametophyte more experimentally tractable than the sporophyte. Second, mutants may be identified shortly after mutagenizing millions of single-celled spores and growing the gametophytes under appropriate conditions. A similar approach has been used to identify mutants and genetically dissect the complex sex-determining pathway in gametophytes of the fern, *Ceratopteris richardii* (Strain et al., 2001), also a member of the Pteridaceae. Finally, a protocol developed in our laboratory for systemically silencing genes in the gametophyte in *C. richardii* gametophytes (Rutherford et al., 2004) is likely to be applicable and useful as a reverse genetics tool in *P. vittata* for studying gene function.

As previous studies of As accumulation and tolerance in *P. vittata* have focused entirely on the sporophyte, the purpose of this study was to determine if and to what extent the *P. vittata* gametophyte is also able to tolerate and accumulate As. Gametophytes of other members of the Pteridaceae, *Pityrogramma calomelanos*, and *C. richardii*, were included in this study for comparative purposes. *Pityrogramma calomelanos* sporophytes accumulate and tolerate As but at levels lower than that reported for *P. vittata* (Francesconi et al., 2002). *C. richardii* was included because it is an established model system for genetic analyses of gametophytically expressed traits (Strain et al., 2001), and is an As nonaccumulator. The results of this study show that gametophytes of *P. vittata*, like the sporophyte, tolerate and accumulate As when grown in the presence of As, compared to gametophytes of *C. richardii* which fail to germinate in the presence of As. Furthermore, gametophytes of *P. calomelanos* display an intermediate phenotype in As tolerance and accumulation when compared to gametophytes of *P. vittata* and *C. richardii*. In surveying natural populations of *P. vittata*, we have also identified an individual that has a reduced ability to tolerate As. How these results can be used to genetically dissect the mechanisms underlying As tolerance and accumulation in *P. vittata* are discussed.

RESULTS

As Tolerance and Accumulation in Gametophytes of *P. vittata*, *C. richardii*, and *P. calomelanos*

As accumulation and tolerance in *P. vittata* gametophytes was measured to determine if the small,

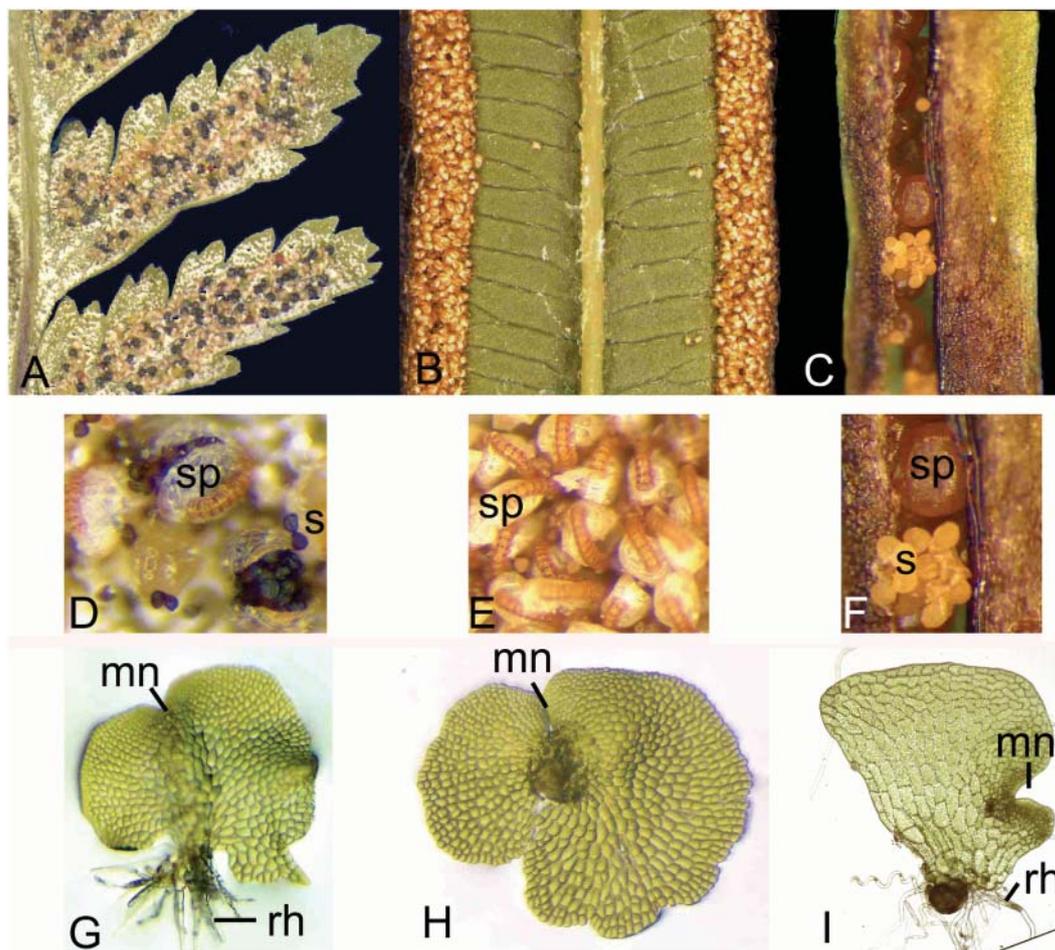


Figure 1. The spore-forming and gamete-forming phases of the fern life cycle. A, The underside of a diploid *P. calomelanos* sporophyll showing numerous sporangia containing the haploid spores. B, The underside of a *P. vittata* sporophyll. C, The underside of a *C. richardii* sporophyll. D, *P. calomelanos* sporangia and black spores. E, *P. vittata* sporangia and spores. F, *C. richardii* sporangia and spores. G, The haploid *P. calomelanos* gametophyte has numerous rhizoids and a meristem notch. H, The haploid *P. vittata* gametophyte showing the meristem notch. I, A hermaphroditic *C. richardii* gametophyte with numerous rhizoids and a meristem notch. s, sporangium; sp, spore; mn, meristem notch; rh, rhizoid. Bar represents 0.5 mm.

free-living gametophytes of *P. vittata* are able to tolerate and accumulate As. Spores from a single sporophyte plant were harvested, surface sterilized, and grown in liquid media containing varying concentrations of arsenate. As tolerance is defined here as the ability of the gametophyte to grow in the presence of As and was measured by determining the dry weight of each gametophyte population after 1 month of growth. As shown in Figure 2, gametophyte growth was not significantly reduced until arsenate concentrations in the medium exceeded 5 mM. At 15 mM arsenate, growth was reduced approximately 40% compared to control gametophytes grown in the absence of As (Fig. 2A). The morphology of gametophytes grown on agar solidified medium containing varying concentrations of As, also an indicator of As tolerance, is illustrated in Figure 3, A to E. The size and morphology of *P. vittata* prothalli are indistinguishable when grown in the presence of no or 1 mM arsenate (Fig. 3, A

and C). At lower concentrations of arsenate (0.2 mM), the prothalli are typically larger compared to the no arsenate control (compare Fig. 3, A and B), while at higher arsenate concentrations (>1 mM), the overall prothallus size decreases (Fig. 3, D and E). Although the shape and cellular organization of the prothallus remains relatively unchanged in the presence of arsenate, the size and number of rhizoids that emerge from the prothallus increases as the concentration of As increases in the media. Rhizoids are particularly evident on gametophytes grown in medium containing 10 mM and 15 mM arsenate, as illustrated in Figure 3, D and E.

The amount of As accumulated by the same gametophyte populations used to measure changes in dry weight in the presence of varying concentrations of arsenate was determined by inductively coupled plasma mass spectrometry (ICP-MS). As shown in Figure 2B, *P. vittata* gametophytes accumulate more

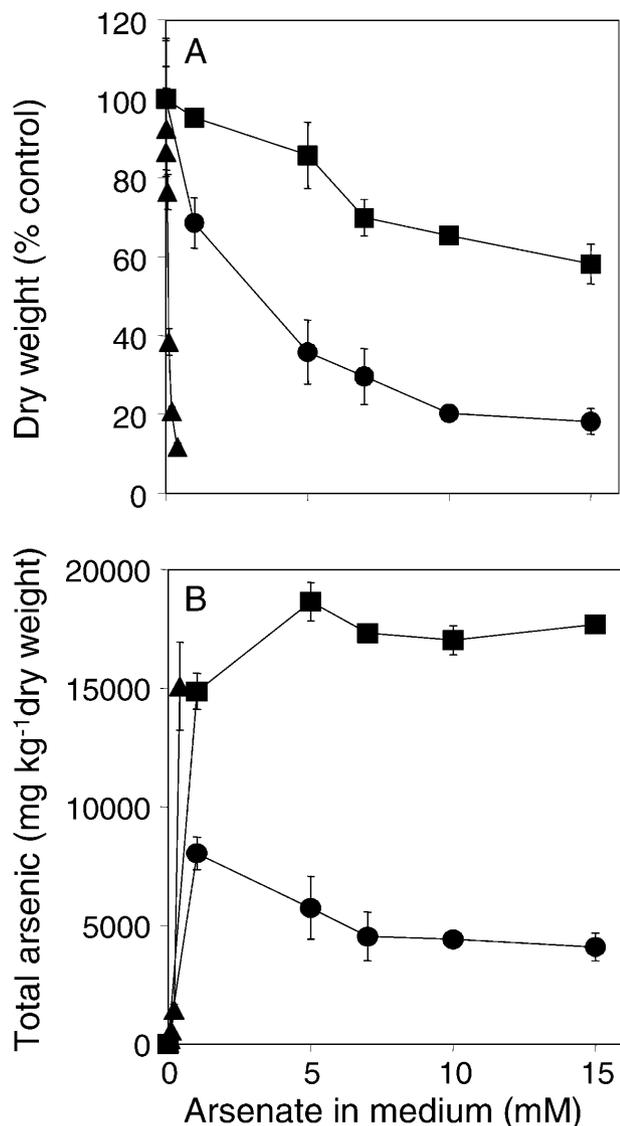


Figure 2. As tolerance and accumulation in *P. vittata*, *C. richardii*, and *P. calomelanos*. Gametophytes of *P. vittata* (squares), *P. calomelanos* (circles), and *C. richardii* (triangles) were grown for 1 month in liquid culture, harvested, and total dry weight (A) and As content (B) measured. Data represents the average (\pm SE) of three replicate cultures per treatment.

than 15,000 mg As Kg⁻¹ dry weight (or 1.5% of their total dry weight), even when grown in medium containing 1 mM arsenate, a condition where no reduction in growth compared to controls was observed (Figs. 2A and 4). These results demonstrate that *P. vittata* gametophytes hyperaccumulate As to significantly elevated concentrations compared to that present in their surrounding medium.

Gametophytes of *C. richardii*, a relative of *P. vittata*, were also grown and tested for As tolerance and accumulation. Their growth was found to be inhibited by As at concentrations as low as 0.05 mM arsenate in the medium when cultured in liquid media (Fig. 2A).

Severe inhibition of gametophyte development was observed at 0.05 mM arsenate when grown on agar solidified or liquid medium (Figs. 2A and 3Q), and virtually no spore germination or growth observed at 0.2 mM arsenate (Figs. 2A and 3R). At concentrations of As exceeding 0.4 mM, *C. richardii* spores grown in liquid medium accumulate high concentrations of As (15,000 mg As Kg⁻¹ tissue; Fig. 2B); however, this accumulation of As is not biologically relevant since As is clearly toxic to the gametophyte at the same concentrations (Fig. 2A). These results indicate that *C. richardii* gametophytes do not actively accumulate or tolerate As in their surrounding medium.

Gametophytes of *P. calomelanos*, another member of the Pteridaceae whose sporophyte has been shown to accumulate As (Francesconi et al. 2002), were also tested for As tolerance. Gametophytes of *P. calomelanos* grown in liquid medium were observed to display a relatively high level of tolerance to arsenate compared to *C. richardii*, with significant reductions in growth observed only when concentrations of arsenate in the medium exceeded 1 mM (Fig. 2A). Gametophytes grown on agar-solidified medium containing low concentrations of arsenate (0.2 mM) were typically larger than gametophytes grown in the absence of arsenate (Fig. 3, K and L). A reduction in prothallus size was observed when concentrations of arsenate exceeded 1.0 mM. This reduction in size was associated with a change in the shape of the prothallus, from heart-shaped to filamentous (Fig. 3, K–O). The filamentous morphology of gametophytes indicates that As inhibits the transition from one- to two-dimensional growth of the prothallus, a developmental transition that is typical of most homosporous fern gametophytes. While arsenate inhibits two-dimensional growth of the prothallus, the number and length of rhizoids per gametophytes increased when gametophytes were grown on agar solidified medium containing As. This stimulation of rhizoid growth by arsenate was evident when gametophytes were grown on as low as 0.2 mM arsenate (Fig. 3L).

To test the ability of *P. calomelanos* gametophytes to accumulate As, the amount of As in the same gametophyte populations used to determine changes in dry weight (Fig. 2A) was assessed. The *P. calomelanos* gametophytes accumulate 8,000 mg As Kg⁻¹ dry weight when grown in medium containing 1.0 mM arsenate (Fig. 2B), a concentration of As that has only a slight inhibitory effect on gametophyte growth when compared to gametophytes grown in the absence of arsenate (Figs. 2A and 3, K and M). Filamentous gametophytes that developed at higher (>1.0 mM) concentrations of arsenate also accumulated high levels of As (approximately 5,000 mg As Kg⁻¹ dry weight). These results demonstrate that *P. calomelanos* gametophytes, like those of *P. vittata*, are able to actively accumulate more As than is present in the surrounding medium.

The comparison of As tolerance (Figs. 2A and 3) and accumulation (Fig. 2B) in gametophytes of *P. vittata*, *C.*

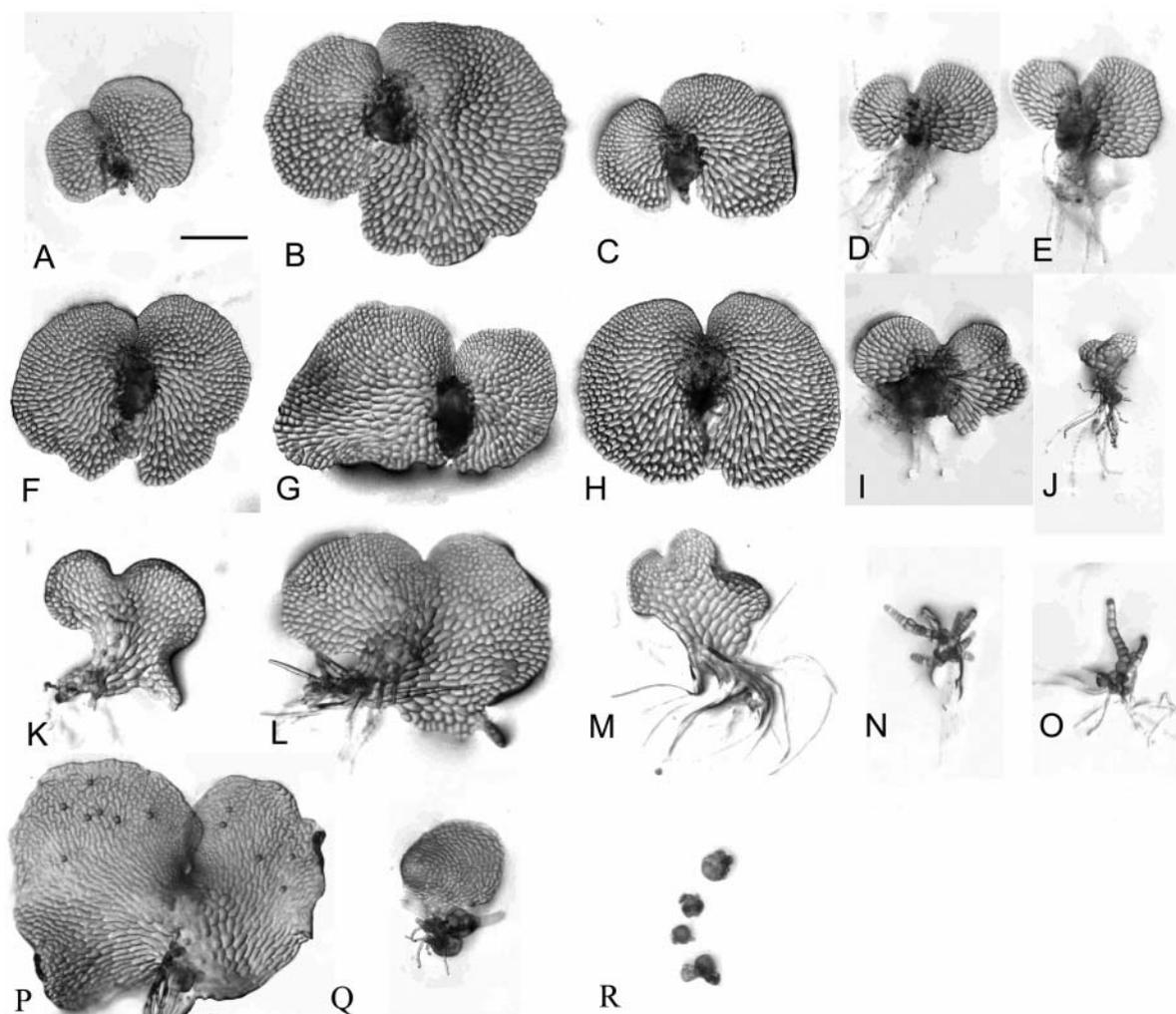


Figure 3. Morphology of *P. vittata*, *C. richardii*, and *P. calomelanos* grown in the presence of varying concentrations of arsenate. Gametophytes were grown as described in Figure 2. A–E, *P. vittata* gametophytes isolated from sporophyte collected at site 4 and (F–J) from sporophyte collected at site 8 grown in liquid medium containing 0 (A), 0.2 (B), 1 (C), 10 (D), and 15 (E) mM arsenate. K–O, *P. calomelanos* gametophytes grown in liquid medium containing 0 (F), 0.2 (G), 1 (H), 10 (I), and 15 (J) mM arsenate. P–R, *C. richardii* gametophytes grown in liquid medium containing 0 (P), 0.05 (Q), and 0.2 (R) mM arsenate. Bar represents 0.5 mm.

richardii, and *P. calomelanos* demonstrate that these As-associated traits vary among species of the Pteridaceae. *P. vittata* and *C. richardii* represent the two extremes, while *P. calomelanos* gametophytes are intermediate in their ability to tolerate and accumulate As.

Phosphate and Arsenate Interactions in *P. vittata* and *P. calomelanos*

Arsenate is an analog of phosphate and is likely to be transported via phosphate transporters in the cell. To test the hypothesis that arsenate affects the accumulation of phosphorus (P), the amount of P in *P. vittata* and *P. calomelanos* gametophytes was measured using ICP-MS. Spores of both species were surface sterilized and grown in liquid medium containing 0.73 mM phosphate and varying concentrations of arsenate. After culturing for 1 month, gametophytes were collected,

dried, and analyzed for both P and As by ICP-MS. In both species, the accumulation of P in gametophytes was significantly reduced by the presence of arsenate in the medium, with reductions in P concentrations occurring at both 1 and 5 mM arsenate (Fig. 4, A and B). However, P concentrations in the gametophytes were found to stabilize at 7 mM external arsenate and remained constant up to 15 mM arsenate (Fig. 4, A and B). The rapid drop in P concentrations coincides with a rapid increase in accumulated As in both species (Fig. 4, A and B), and the stabilization of P concentrations coincided with stabilization of the concentration of accumulated As (Fig. 4, A and B). However, the reduction in P observed in *P. calomelanos*, though similar to *P. vittata* in magnitude, occurs at much lower internal As concentration. Such differences in P status may reflect alterations in the underlying mechanisms of As homeostasis in these species.

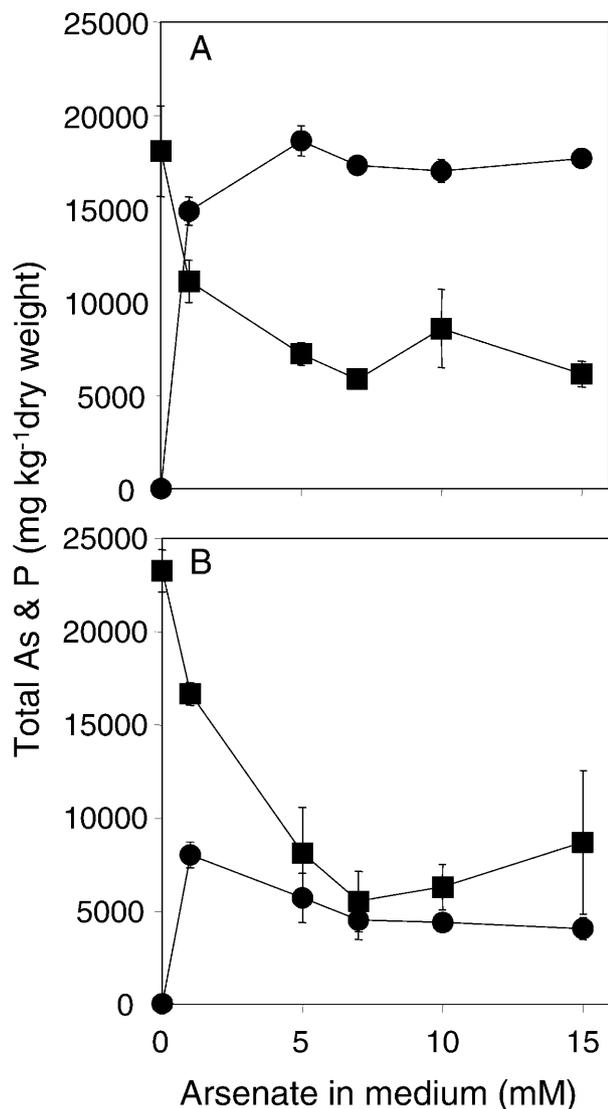


Figure 4. Phosphorus and As accumulation in *P. vittata* and *P. calomelanos*. Gametophytes of *P. vittata* (A) and *P. calomelanos* (B) were grown as described in Figure 2 and total P (squares) and As (circles) measured. Data represents the average (\pm SE) of three replicate cultures per treatment.

As Content in Field Collected *P. vittata* Sporophytes and Soils Is Variable

To assess variation in As tolerance and accumulation among naturally occurring *P. vittata* individuals, 40 intact sporophytes were collected from 16 different sites in Florida (Table I; Fig. 5A) where plants were found growing in diverse habitats, including rock beds, residential properties, and sea walls (Fig. 5, B–E). Total As content in the fronds of plants collected from the field was assessed by ICP-MS and found to be highly variable both within a single plant, between plants collected from the same site, and between plants collected from different sites (Table I). Soluble As in the soil surrounded the roots of the field collected plants was analyzed and also found to vary

within and between sites (Table I). While the amounts of As in plants and soils were variable, the concentrations of As measured in frond tissues tended to reflect the concentration of As in the soil (Table I). Plants and soils collected from sites 5 and 11, for example, had the highest concentrations of As observed in this study (Table I). What distinguishes sites 5 and 11 from all others is that they were potentially contaminated with chromated copper arsenic (CCA), a common wood preservative used to protect exposed lumber from rot. Sporophytes collected from site 5 grew at the base of a retaining wall made of CCA treated lumber. Site 11 was adjacent to a wood treatment facility. The soil at this site is likely to have been contaminated with CCA used during the wood treatment process.

P. vittata plants found growing in soils containing relatively low concentrations of soluble As also accumulated As in their fronds. The ratio of As accumulated in the fronds to the amount of water extractable As in the soil was high in all plants, with fronds accumulating As in the 100s of mg kg^{-1} range from soils containing only $\mu\text{g kg}^{-1}$ As (Table I), indicating that all plants collected are capable of accumulating As at concentrations 2 to 3 orders of magnitude higher than the concentrations of As present in the soil. Altogether, these results indicate that the variation in As accumulation observed within and between field-collected plants is likely to be influenced by the amount of As in the soils and the developmental age of the plant part assessed, but does not eliminate the possibility that genetic variability between individuals may also play a role.

Variation in As Tolerance between *P. vittata* Populations

Having established that *P. vittata* gametophytes can be grown under highly controlled environmental conditions and can tolerate and accumulate As, progeny gametophytes from field collected sporophytes were grown in the presence of varying concentrations of As and examined to see if genetic variation in As tolerance and accumulation exists in this species. Spores from each field-collected plant were harvested and initially grown in liquid medium containing 37 mM arsenate. After 1 month of culture, the dry weight and total As content of each gametophyte population was measured (data not shown). Five gametophyte populations that displayed the greatest differences in either As accumulation or tolerance were selected and tested again for growth and As accumulation in medium containing five different arsenate concentrations. None of these populations showed any significant differences in As accumulation in this second, more stringent selection (data not shown). However, gametophytes grown from spores harvested from a site 8 sporophyte (Table I) showed significantly reduced tolerance to arsenate in the medium when compared to all other gametophyte populations tested, especially

Table I. Sampling location and As levels for 16 field collected *P. vittata* sporophytes

At least 3 replicate plant tissue and soil samples were analyzed for each population as described in "Materials and Methods." As levels presented as ranges.

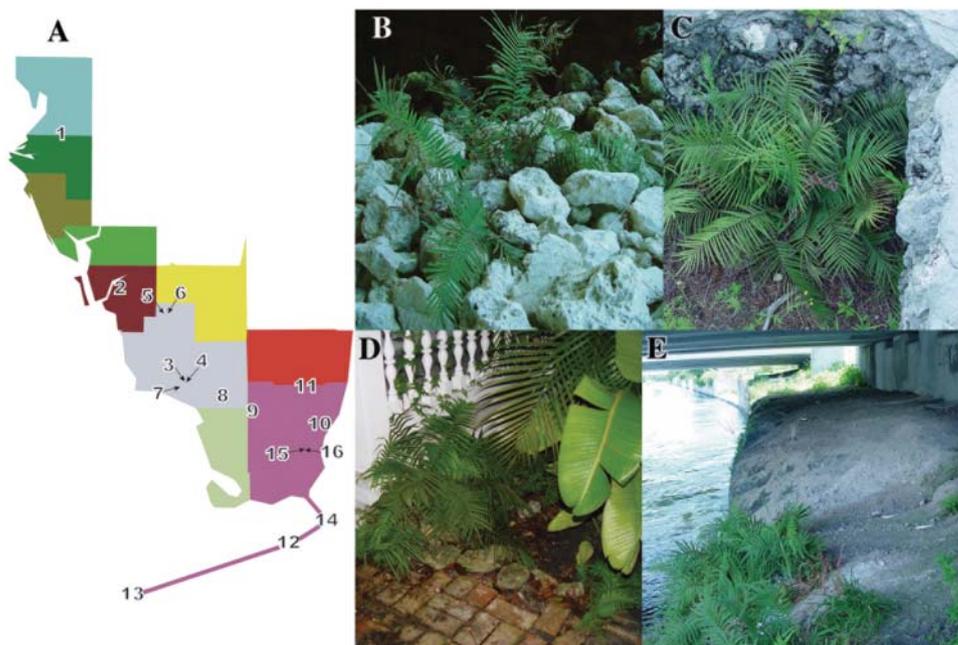
Site	Lat	Long	Number of Individual Sporophytes Sampled per Site	Total Number of Sporophyte Pieces Sampled per Site	Range of Total As in Sampled Frond Tissue (ppm)	Mean of Water Soluble As in Soil (ppb) ^a
1	26.4327	81.4816	6	30	11–588	214
2	25.4815	80.5419	nd ^b	nd ^b	nd ^b	nd ^{b,c}
3	25.9822	81.3489	3	3	51–251	138
4	26.1870	80.1549	1	4	251	nd ^c
5	80.1549	81.4890	1	6	1–2,663	997
6	26.6437	81.8687	2	3	64–385	nd ^c
7	25.9449	81.3599	1	8	6–397	nd ^c
8	25.8650	81.1137	2	8	3–43	nd ^c
9	25.8057	80.8735	2	6	12–42	nd ^c
10	25.8010	80.3233	3	7	49–624	145
11	25.8834	80.3632	6	38	2–3,933	133–7,724 ^d
12	24.9146	80.6400	2	5	4–961	nd ^c
13	24.5554	81.7967	3	27	5–859	210
14	24.5553	81.7967	2	5	4–41	99
15	25.4907	80.5389	3	7	7–153	119

^aOne soil sample was collected for each population and divided into 3 replicates and means presented. ^bOnly spores were collected for this population. ^cSoil was not sampled at these locations. ^dSeven different soil samples were collected at the site and each sample divided into 3 replicates, and the range of the means presented.

when grown at higher (>10 mM) concentrations of arsenate. Gametophytes grown from spores collected from this site 8 plant did not germinate or grow in liquid medium containing 30 mM arsenate, while gametophytes from a site 4 sporophyte, for example, showed little growth reduction even at 40 mM arsenate (Fig. 6A). In comparing the morphology of gametophytes grown on agar-solidified medium containing 15 mM arsenate, substantial differences in the sizes of gametophytes from sites 4 and 8 were also observed,

with site 4 gametophytes being much larger than site 8 gametophytes (Fig. 3, E and J). When grown in the presence of arsenate at concentrations that do not affect the growth of site 8 gametophytes (i.e. 0–10 mM arsenate), no differences in As accumulation between site 4 and 8 gametophytes were observed (Fig. 3B). Site 8 gametophytes did not accumulate As when grown at concentrations of arsenate (>30 mM) that inhibit germination or gametophyte growth. These results indicate that while As accumulation is an invariant trait

Figure 5. Population locations and images of *P. vittata* sporophytes collected throughout south Florida. A, Map of Florida showing locations of sampled populations (for GPS positioning, see Table I). B, Population 4 sporophyte; C, population 8 sporophyte; D, population 13 sporophyte; and E, population 14 sporophyte.



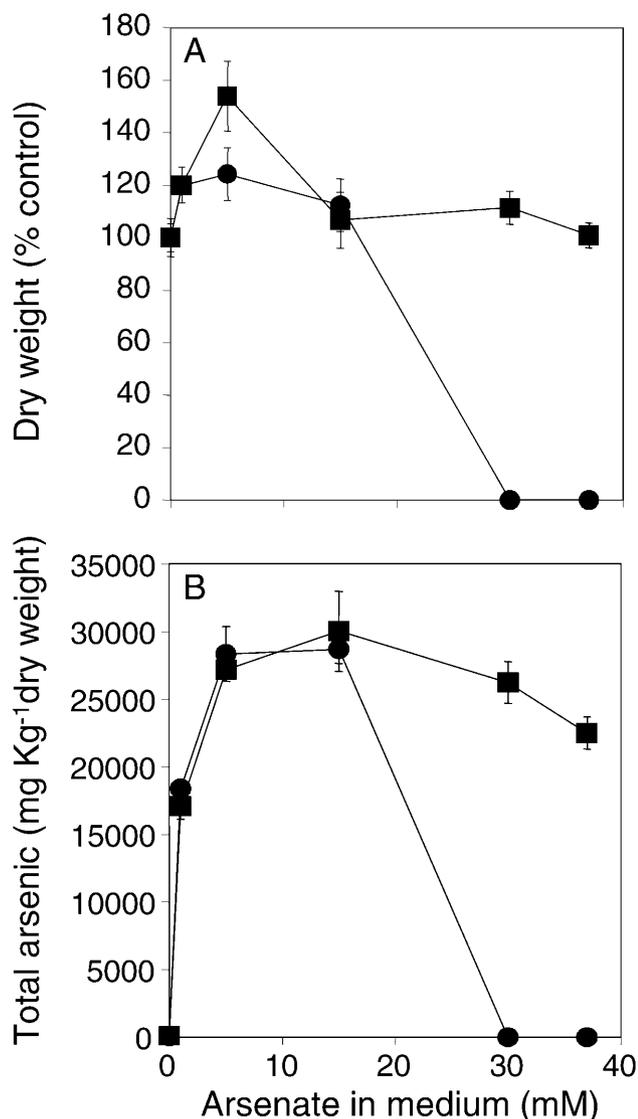


Figure 6. Population variation for As tolerance and accumulation in *P. vittata*. Gametophytes from spores collected from site 4 (squares) and site 8 (circles) were grown liquid medium containing various concentrations of arsenate, and after 1 month growth total dry weight (A) and As accumulation (B) of gametophytes measured. Data represents the average (\pm SE) of three replicate cultures per treatment.

in the gametophytes examined, tolerance to As does vary in natural populations of *P. vittata*.

DISCUSSION

Numerous studies conducted during the past 5 years have demonstrated that the *P. vittata* sporophyte is very unusual in its ability to tolerate and accumulate very high concentrations of As (Ma et al., 2001; Chen et al., 2002; Visoottiviseth et al., 2002), a potentially toxic contaminant of soils and groundwater in many regions of the world. The lack of forward- or reverse-genetic tools in *P. vittata* and the slow-growing nature

of the *P. vittata* sporophyte severely limit the ability to identify and study the genetic or molecular factors that make this plant unique. One unexplored means to study traits in this species is to take advantage of the gametophyte, which represents the haploid, gamete-producing phase of the plant life cycle. Unlike the gametophytes of flowering plants, which are dependent upon and surrounded by sporophytic tissues of the flower, the gametophytes of homosporous ferns are very small yet free-living. Because they are also haploid and fast-growing, fern gametophytes can be grown under highly controlled conditions while mutations that perturb gametophyte growth and development can be easily and quickly selected then genetically characterized (Banks, 1999). One purpose of this study was to determine whether the *P. vittata* gametophyte is also able to tolerate and accumulate high concentrations of As.

Sporophyte fronds of *P. vittata* harvested from 40 field-collected plants from 16 sites in Florida were found to accumulate up to 0.39% of their total dry weight as As, similar to levels previously reported for *P. vittata* sporophytes (Ma et al., 2001; Wang et al., 2002). All of the *P. vittata* sporophytes examined accumulated As in the parts per million (ppm) range yet grew in soils having as little as 100 parts per billion (ppb) As, indicating that all of the sporophytes sampled have the potential to hyperaccumulate As from the soil. Progeny gametophytes of these *P. vittata* sporophytes were found to accumulate up to approximately 2.5% of their dry weight as As when grown in medium containing 5 to 10 mM arsenate, demonstrating that their gametophyte progeny are also able to hyperaccumulate As from their surrounding medium. Tolerance to As, assessed by measuring the dry weight of gametophytes grown in media containing As, was also evident in *P. vittata* gametophyte populations. Based on studies by Wang et al. (2002), loss of *P. vittata* sporophyte biomass occurs when concentrations of As exceed 0.5 mM. With one exception, the dry weight of *P. vittata* gametophyte populations studied here did not begin to diminish significantly until As concentrations exceed 5 mM, indicating that the gametophyte is able to tolerate even higher concentrations of As than the sporophyte.

Previous studies have shown that As tolerance and accumulation is not unique to *P. vittata*. Sporophytes of other members of the Pteridaceae, including *Pteris cretica*, *Pteris longifolia*, *Pteris umbrosa* (Zhao et al., 2002), and *Pityrogramma calomelanos* (Francesconi et al., 2002) are able to tolerate and accumulate very high levels of As. The gametophytes of three members of the Pteridaceae, including *P. calomelanos* and *C. richardii*, were examined in this study. *C. richardii* was included because it has been developed as a model genetic system and reverse genetics tools for studying the function of gametophytically expressed genes have been developed in our laboratory (Rutherford et al., 2004). *C. richardii* gametophytes were found to be very sensitive to exogenous As in the growth medium and did not accumulate As. *P. calomelanos* gametophytes

were found to accumulate and tolerate As, but at levels lower than that observed in *P. vittata*. This intermediate phenotype for As-associated traits also has been observed in the sporophytes of *P. calomelanos* (Francesconi et al., 2002). In addition to affecting the dry weight of the gametophyte population at high concentrations, As was also found to stimulate rhizoid growth but inhibit two-dimensional growth of the *P. calomelanos* gametophyte. Whether As directly or indirectly influences these traits is not known at this time.

Having three species of related ferns that vary in their ability to tolerate and hyperaccumulate As is invaluable for genetically dissecting the mechanisms underlying these traits using the gametophyte as a target for selection. Based upon what is known about the physiological and morphological responses to As in each species, future experiments can be easily designed to select mutant *P. vittata* gametophytes that are arsenate intolerant, *C. richardii* gametophytes that are arsenate tolerant, and *P. calomelanos* gametophytes that have reduced or enhanced tolerance to arsenate. Controlled crosses between mutants in each species can be used to determine the heritability of a mutant phenotype, the number of gene loci that contribute to each trait, and, by comparing phenotypes of mutant gametophytes and sporophytes heterozygous or homozygous for each mutation, determine if As-associated traits are regulated by similar mechanisms in the gametophyte and sporophyte phases of development. Because the gametophyte is very simple in its morphology and lacks the complex roots, leaves, stems, and vascular system of the sporophyte, the gametophytes of all three species will serve as useful experimental systems for comparing and understanding how As is (or is not) metabolized and localized in cells of each species.

Another approach that is useful for identifying and ultimately cloning genes involved As tolerance and accumulation in *P. vittata* is to identify individuals, or ecotypes, of *P. vittata* that show heritable differences in their ability to tolerate or accumulate As. Once identified, crosses between ecotypes and testing progeny gametophytes for segregation of As-associated traits can be used to identify the genes underlying these differences. In surveying gametophytes generated from 40 sporophyte plants collected from the wild, we have observed no differences in As accumulation, although the progeny gametophytes of one sporophyte plant was found to exhibit decreased tolerance to As when grown at high levels of arsenate. Crosses between this As-intolerant gametophyte and an As-tolerant gametophyte are currently being performed. DNA polymorphisms that cosegregate with As-intolerance will be sought and may be used to clone the gene(s) of interest. Given that *P. vittata* is a species native to China and was only introduced into the United States in the 1920s (Maxon, 1926), genetic variation among *P. vittata* plants that grow in the United States may be quite low due to founder effects. Efforts to determine genetic variability in natural populations using DNA markers are currently under way.

Potential mechanisms involved in As tolerance and accumulation in *P. vittata* can be gleaned by comparing the physiological responses to As in different members of the Pteridaceae. Arsenate is a chemical analog of phosphate and previous experiments have shown that arsenate competes with phosphate for uptake in plants (Meharg and Macnair, 1990, 1991, 1992; Meharg et al., 1994; Meharg and Hartley-Whitaker, 2002). Increasing external phosphate concentrations while maintaining constant arsenate increases arsenate tolerance (Meharg and Macnair, 1991). Heritable modification of phosphate transporters that reduce arsenate uptake appear to be responsible for arsenate tolerance in certain ecotypes of *Holcus lanatus* (Macnair and Cumbes, 1987; Meharg and Macnair, 1991). Arsenate and phosphate also appear to compete for uptake in *P. vittata* sporophytes (Wang et al., 2002). Moreover, fluctuations in external arsenate concentration reduce internal P, and increased external phosphate leads to decreased internal As (Wang et al., 2002). Such competition between arsenate and phosphate for uptake suggests that *P. vittata* may lack a specific arsenate transporter, and instead arsenate is taken up by phosphate transporter(s). In *P. vittata* gametophytes we have observed that increasing external levels of arsenate, while keeping phosphate constant, also leads to a drop in internal P levels, similar to that observed in the sporophyte. This drop in internal P appears to be proportional to the accumulation of As, suggesting that arsenate and phosphate are transported via the same pathway. In contrast, *P. calomelanos* gametophytes do not appear to have a proportional response to external As and P. Rather, levels of P drop rapidly with the addition of only low concentrations of As. Interestingly, concentrations of Ca, Mg, Mn, Mo, and Zn in *P. vittata* and *P. calomelanos* (data not shown) do not fluctuate in response to increasing levels of external As suggesting that the different As/P interactions observed in *P. vittata* and *P. calomelanos* are not related to nonspecific arsenate toxicity. The differences in As/P interactions may reflect specific differences in the mechanism(s) used to accumulate and tolerate As. One possible difference would be that arsenate uptake in both *P. vittata* and *P. calomelanos* is via a phosphate transporter, however, As tolerance in *P. calomelanos* is achieved by efflux, similar to yeast and *Escherichia coli* (Rosen, 2002), leading to equal reductions in phosphate between species but much lower accumulation on As in *P. calomelanos*. Further research is required to resolve these possibilities.

CONCLUSION

Here we present data, showing for the first time, to our knowledge, that *P. vittata* gametophytes tolerate and hyperaccumulate As to a similar extent to that observed previously in the sporophyte of this species. Given the simple growth habit and haploid nature of the gametophyte, such observations open the door to

the application of new and powerful forward- and reverse-genetic tools for the dissection of the molecular mechanisms involved in As hyperaccumulation in *P. vittata*. Identification of genes involved in As hyperaccumulation in ferns will provide valuable genetic resources for the future development of plants ideally suited for As phytoremediation.

MATERIALS AND METHODS

Sporophyte and Gametophyte Culture

Pteris vittata sporophytes were collected in September 2002 from 16 sites in Florida listed in Table I. All plants were transferred and grown in a 1:1 mixture of topsoil and potting soil and maintained in greenhouses at Purdue University. Sporophylls from each plant were placed in glassine bags for 2 weeks; spores released within each bag were collected and stored at room temperature. The origin of Hn-n, the *Ceratopteris richardii* strain used in these studies, is described previously (Scott and Hickok, 1987). *Pityrogramm calomelanos* spores were collected from a sporophyte plant obtained from Duke University. All spores were surface sterilized by soaking in solution containing 50% bleach and 0.5% Tween 20 for 4 min and washing four times in sterile water. Medium for culturing gametophytes contained 0.5× MS salts (Sigma M5524, St. Louis), pH 6.5. When required, medium was solidified with 0.65% agar (Sigma A9915) prior to autoclaving. Arsenate stock solutions were prepared for potassium arsenate monobasic anhydrous (Sigma A6631) dissolved in 18 MΩ water, filter sterilized through a 0.2 μm cellulose acetate filter, and where necessary added to previously autoclaved medium. Liquid cultures were placed on an orbital shaker table, and all cultures grown at 24°C to 27°C under continuous illumination (41–52 μmol m⁻² s⁻¹ photosynthetic photon flux) provided by cool-white fluorescent bulbs.

Sample Preparation and Elemental Analysis

Soil surrounding the roots of field-collected plants was removed and stored at 4°C for 8 months prior to elemental analysis. Three 1 g aliquots of oven-dried soil were suspended and agitated for 14 h in 20 mL of 18 MΩ water. Soil suspensions were filtered through a Whatman filter (No. 2) and 15 mL of filtrate placed into clean borosilicate glass test tubes. Water was evaporated and residues analyzed by ICP-MS. Pieces of fully expanded pinnae and fronds from field-collected sporophytes were sampled randomly from individual plants 4 weeks after collection from the field. Tissue from each plant was subdivided into three, dried for 24 h at 80°C, weighed, placed into borosilicate glass test tubes, and analyzed by ICP-MS. Gametophytes were either grown in liquid or on agar solidified media for 30 d prior to harvesting. In all experiments, spores were sterilized and aliquoted into flasks containing liquid media, such that each replicate contained equal numbers of spores. Liquid grown and agar-solidified medium grown gametophytes were filtered over a preweighed Whatman filter (No. 3), washed with 10 mL of 50 mM potassium phosphate buffer (pH 7.0) to desorb cell wall associated arsenate, and extensively with 18 MΩ water. Filters containing gametophytes were dried for 48 h at 72°C. Each filter with its associated gametophyte population was weighed, and initial filter weight subtracted to determine final gametophyte dry weight. For ICP-MS analyses 50 mg of gametophyte tissue was weighed into a triple rinsed borosilicate glass test tube for elemental analysis.

All soil and plant samples were acid digested with 1.5 mL of concentrated nitric acid (Fisher TraceMetal grade) for 4 h at 118°C. Samples were diluted with 8.5 mL 18 MΩ water and analyzed three times using a Thermo Elemental PQ ExCell ICP-MS. ICP-MS analytical conditions were achieved as previously described (Lahner et al., 2003) to fully quantify antimony, As, bismuth, magnesium, manganese, and phosphorous.

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