High As exposure induced substantial arsenite efflux in As-hyperaccumulator *Pteris vittata*

Yanshan Chen, Jing-Wei Fu, Yong-He Han, Bala Rathinasabapathi, Lena Q. Ma

*State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Jiangsu 210023, China*

**Highlights**
- AsIII efflux was tested at different As concentrations under sterile condition.
- High As exposure induced substantial AsIII efflux in As-hyperaccumulator *Pteris vittata*.
- AsIII efflux may help As detoxification in plants under high As exposure.
- AsIII efflux may not negatively affect As hyperaccumulation in *P. vittata*.

**Abstract**
Arsenite (AsIII) efflux is an important mechanism for arsenic (As) detoxification in plants. Low AsIII efflux has been observed in As-hyperaccumulator *Pteris vittata*, which may contribute to its highly efficient As translocation and accumulation; however, the results may be compromised by microbial AsIII oxidation, relatively low As concentration in the medium and short-term As exposure. Here, sterile *P. vittata* sporophytes were cultivated in sterile medium containing 10, 200 and 500 μM arsenate (AsV) for 28 d. Arsenite efflux to the growth medium and As speciation in *P. vittata* was investigated. Low AsIII efflux at 12% of AsV uptake was observed at 10 μM AsV, but high AsIII efflux (36–76%) was observed at 200 and 500 μM AsV, with 1987–2397 mg kg⁻¹ As being accumulated in the fronds. This is the first report to show efficient AsIII efflux in *P. vittata*. This study showed that *P. vittata* may use high AsIII efflux to cope with As toxicity under high As exposure, which may be necessary to sustain growth while accumulating As.

**1. Introduction**
Arsenic is ubiquitous in soils and toxic to humans. It often presents in two oxidation states: arsenate (AsV) and arsenite (AsIII), with AsIII being more toxic and mobile than AsV. To survive under arsenic-rich environment, organisms have evolved various As-detoxification mechanisms. One important mechanism in microbes is to efflux AsIII from cytosol to the external environment. In both prokaryote *Escherichia coli* and eukaryote *Saccharomyces cerevisiae*, As detoxification involves reduction of AsV to AsIII and...
subsequent AsIII extrusion to the external medium (Rosen, 1999; Ghosh et al., 1999).

Similar strategy has also been adopted by plants. Xu et al. (2007) found that after taken up by tomato and rice in the roots, AsV is rapidly reduced to AsIII and effluxed to the external medium. It is reported that Lsi1 (OsNIP2; 1, an aquaporin channel) plays a role in AsIII efflux in rice roots. However, it accounts only part of the efflux, suggesting the presence of other efflux transporters (Zhao et al., 2010). Since AsIII enters plant roots via aquaporins, which transport AsIII bidirectionally across membranes (Ali et al., 2009; Bienert et al., 2008), it is possible that besides Lsi1, some aquaporins are involved in AsIII efflux in plants. The AsIII efflux transporter ACR3 from S. cerevisiae and Pteris vittata has been demonstrated to increase AsIII efflux when expressed in plants (Ali et al., 2012; Duan et al., 2012; Chen et al., 2013). After expressing PVA3CR3 in Arabidopsis, AsIII efflux significantly increased and the plants showed enhanced As tolerance, indicating that AsIII efflux confers As detoxication in plants (Chen et al., 2013). Although effluxed AsIII in the medium can be reabsorbed by plant roots, continuous efflux decreases As accumulation over time and reduces As toxicity in plants (Zhao et al., 2009).

P. vittata, the first known As hyperaccumulator, is extremely efficient in As uptake (Ma et al., 2001). Since P. vittata can grow in highly As-contaminated soil, studying AsIII efflux in P. vittata under high As treatment may help to understand its unique As metabolism pattern. Compared to non-hyperaccumulators, P. vittata shows a low AsIII efflux activity, consistent with its highly efficient AsIII xylem transport and high accumulation in the fronds (Su et al., 2008; Huang et al., 2011). However, those studies on AsIII efflux in both non-As-hyperaccumulating (Xu et al., 2007; Zhao et al., 2010; Duan et al., 2012; Ali et al., 2012; Chen et al., 2013) and As hyperaccumulating plants (Su et al., 2008; Huang et al., 2011) used relatively low AsV concentrations (5–20 μM or 0.375–1.5 mg/L). Though they represent typical concentrations observed in the environment, they may be insufficient to investigate the role of AsIII efflux in As detoxication in As hyperaccumulating plants in contaminated environment. In addition, all studies were conducted in non-sterile environment where microbial As transformation is unavoidable (Rhine et al., 2005; Wang et al., 2012). Since P. vittata is highly tolerant to As (up to 500 μM) (Gumaelius et al., 2004; Yang et al., 2007) and thrives in As-rich soil (Xu et al., 2014), it is necessary to characterize AsIII efflux under a range of AsV concentrations under sterile condition.

To achieve this goal, we performed a long-term study to examine AsIII efflux of P. vittata under sterile conditions by determining As speciation in the medium and plant after exposing 10, 200 or 500 μM AsV for 28 days. We hypothesized that the occurrence of AsIII in the medium was probably from AsIII efflux by P. vittata roots. The result may help to understand the balance between As uptake and efflux, and As accumulation and detoxication in As hyperaccumulator P. vittata under different As stress.

2. Materials and methods

2.1. Gametophyte and sporophyte culture

Sporophytes of P. vittata were collected from Florida, USA and they were surface-sterilized by immersing them in 0.2% Triton X-100 for 10 min followed by 0.5% NaClO for 15 min. After washing with sterile water, the spores were sown on 1/2 Murashige and Skoog (MS) agar medium containing 0.8% agar at pH 5.8 (Yang et al., 2007). Gametophytes were germinated after 20 d, and after 20 d of growth, they were transferred to fresh 1/2 MS solid medium. After ~2 months of cultivation, sporophytes appeared. At 2–3 fronds stage, the sporophytes were transferred to fresh 1/2 MS medium every 2 months. After growing 7–9 months, sporophytes with 8–10 fronds were used in the experiments. All vessels were placed in a growth chamber at 22 °C with a 16-h light/8-h dark to facilitate germination and growth under light intensity of ~12,000 LX.

2.2. As determination and speciation in the medium and plant

Sporophytes with 8–10 fronds were transferred into 0.6 L bottles (1 plant per bottle) containing 100 ml 1/2 MS agar medium with 0.4% agar at pH 5.8, supplemented with 10, 200 or 500 μM AsV as NaH₂AsO₄·7H₂O. Medium containing 10 μM AsV with no plant was set as a control with three replicates for each treatment. During the growth, aliquots of 0.2-ml nutrient solution were taken after 0, 7, 14, 21 and 28 d and diluted for analysis of total As and As species. The agar used here was about half of the normal concentration, making it easier to collect samples using a micropipette. All experiments were conducted in a bench under clean air to maintain sterile condition and no bacterial contamination was observed during the cultivation. After 28 d of growth, P. vittata plants were harvested and freeze-dried for 48 h for both total As concentration and As speciation analysis.

Total As concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS). For total As concentration in the medium, the nutrient solution was diluted 40–2000 times using 0.1 M HNO₃ to achieve As concentrations within the calibration curve concentrations of ICP-MS (1–20 ppb). For total As concentration in the P. vittata, plant tissues were digested with 50% HNO₃ at 105 °C, following USEPA Method 3050B (Xu et al., 2014). For quality assurance and quality control (QA/QC), indium was used as internal standards and was added into the samples, calibration standards, and blanks. During measurement, standard solution at 5 ppb As was measured every 20 samples to monitor the stability of ICP-MS. The check recovery was within 90–110%. In addition, blanks and certified reference material for plant samples (GB 21, Chinese geological reference materials) were included for quality assurance, which were within expected values.

As speciation was measured using high performance liquid chromatography coupled ICP-MS (HPLC-ICP-MS). For As speciation in the medium, nutrient solution was diluted 40–2000 times using Milli-Q water to achieve As concentrations within 1–20 ppb, following by filtration through 0.22 μm filters. For P. vittata, ground freeze-dried plant sample was ultrasonically extracted with 50% methanol following Xu et al. (2014). Inorganic AsIII and AsV were separated by an anion exchange column (PRPX100, 10 mm, Hamilton, UK) fitted with a guard column (Hamilton, UK). Quality assurance was obtained through the blanks, standard curves, and spiked samples. AsIII and AsV were predominant in the medium and in plant tissues, other As species were not detected in this study.

3. Results and discussion

3.1. Arsenic at 10–500 μM AsV promoted the growth of P. vittata

To better understand AsIII efflux in P. vittata, we used sterile P. vittata under sterile condition to eliminate the effect of microbes on As transformation. In this study, uniform P. vittata sporophytes grew in the medium containing 0, 10, 200 or 500 μM AsV (0, 0.75, 15 or 37.5 mg/L) under sterile condition for 28 d. Even though we used relatively high As concentration at 500 μM AsV, after 28 d of growth, there was no significant difference in biomass, indicating no As toxicity to P. vittata (Fig. 1A). For example, the biomass of P. vittata increased 13.6–14.3% at 10, 200 and 500 μM AsV, which was higher than the 8.8% increase in the no As control (Fig. 1B).
These results indicated that 10–500 μM AsV in the medium caused no toxicity to P. vittata, on the contrary, it promoted the growth of P. vittata.

Our results were in agreement with a previous study showing As enhances P. vittata growth (Gumaelius et al., 2004). For non-hyperaccumulators, AsV at >200 μM is highly toxic. For P. vittata, however, 200–500 μM AsV is tolerable and promotes its growth (Yang et al., 2007). For example, at <1 mM AsV, the growth of P. vittata gametophytes and callus increased compared to the no AsV control (Gumaelius et al., 2004; Yang et al., 2007).

3.2. Arsenite was observed in the growth medium in presence of P. vittata

During the 28 d of growth, no sign of microbial growth in the medium was observed. Since AsV was supplied under sterile condition, it should remain as AsV. Since it has been shown that no reductant is present in the medium and root exudates have little effect on AsV reduction (Xu et al., 2007), we hypothesized that AsIII in the medium was probably from AsIII efflux by plant roots followed AsV uptake and reduction in the root cells.

Our results were consistent with our hypothesis. In the control with 10 μM AsV and no plant, As concentration remained unchanged with little AsIII being detected in the medium during the 28 d of experiment (Fig. 2A). Our data indicated that AsV was stable in the sterile medium. However, in the presence of P. vittata, As concentration in the medium deceased while AsIII increased with time (Fig. 2B–D), which was attributed to As uptake and AsIII efflux by P. vittata. Research shows that root exudates of P. vittata contribute little to AsV reduction (Xu et al., 2007). During the 28 d of growth, the agar medium may have become anaerobic (Chung and Fert, 1999), however, it didn’t lead to the production of AsIII.

Fig. 1. Biomass and the growth of P. vittata sporophytes during As exposure. Statistical analyses of fresh weight (FW) at 0 d and 28 d (A) and growth of P. vittata calculated as the ratio of FW increase during 28 d As exposure to that at 0 d (B). Data represent the mean of three replicates with standard error, and significant differences were established by using one-way analysis of variance (ANOVA).

Fig. 2. Changes in total arsenic (As; closed circle), arsenate (AsV; closed triangle) and arsenite (AsIII; open circle) concentrations in the medium during 28 d growth of P. vittata. No plant (A) and uniform 10-month-old P. vittata sporophytes (B–D) were transferred into 100 ml agar medium supplemented with 10 μM (A and B), 200 μM (C) or 500 μM (D) AsV. Total As and As species were detected by ICP-MS and HPLC-ICP-MS, respectively. Error bars are mean ± SE of three replicates.
Even in an anaerobic environment, it is the microbes who play an important role in mediating As reduction (Rhine et al., 2005). This was further confirmed in the control of 200 or 500 μM AsV with no P. vittata, the result was similar to 10 μM AsV with As remaining constant and little AsIII being detected (<1.0%, data not shown).

However, in the presence of P. vittata, As concentrations in the medium were decreased due to As uptake by P. vittata. In addition, the decrease in As concentrations in the medium was accompanied by increase in AsIII, probably resulting from AsIII efflux from the roots. For example, in 10 μM AsV treatment, total As concentration in the medium decreased from 709 to 87 ppb after 28 d of growth (Fig. 2B). P. vittata is highly efficient in As uptake (Ma et al., 2001), which was consistent with the rapid As depletion in the medium. However, most of the As in medium was present as AsIII, which was 74 ppb, accounting for 85% of the total As remaining in the medium and 10% of 10 μM AsV supplied. The data suggested that AsIII was probably effluxed from the roots of P. vittata after AsV uptake.

In this study, with increasing AsV in the medium from 10 to 200–500 μM, AsIII in the medium increased to 4.30 and 26.1 ppm after 28 d of growth, which was 68% and 87% of total medium As and 30% and 70% of the initial AsV supplied. The data suggested that the increase of As concentration in the roots was linear but not that in the fronds, which may suggest that As translocation may become a limiting factor at high As exposure. However, for all treatments, arsenic concentrations in the fronds were 1.3–2.3 fold higher than those in the roots (Fig. 3A), typical of As hyperaccumulators.

3.3. Arsenate dominated in the roots and arsenite in the fronds of P. vittata

Though AsIII efflux increased under higher AsV exposure, arsenic accumulation in P. vittata was increased too. The arsenic concentrations in the roots and fronds in 10 μM AsV treatment were 47.8 and 111 mg kg−1, which increased to 1803 and 2397 mg kg−1 at 500 μM AsV (Fig. 3A). The results indicated that the increase of As concentration in the roots was linear but not that in the fronds, which may suggest that As translocation may become a limiting factor at high As exposure. However, for all treatments, arsenic concentrations in the fronds were 1.3–2.3 fold higher than those in the roots (Fig. 3A), typical of As hyperaccumulators.

Similar to previous study, AsV dominated in the roots and AsIII in the fronds of P. vittata. As expected, arsenic was mainly present as inorganic forms with little detectable organic arsenic (Ma et al., 2001). The percentage of AsIII was much higher in the fronds (81–92%) than the roots (12–24%) (Fig. 3B). It is possible that both AsIII efflux into the external medium and AsIII xylem transport resulted in low AsIII in the roots, thereby reducing AsIII toxicity to the roots (Xu et al., 2007).

3.4. Arsenite efflux by P. vittata increased exponentially under high As exposure

To compare efficiency of AsIII efflux in different plants, AsIII efflux activity was calculated from AsIII production in medium based on root FW or as a percentage of AsV uptake. For tomato, AsIII efflux was 61–67 μg g−1 root or 64–69% of As uptake (Xu et al., 2007); for rice, it was 68–72 μg g−1 root or 50–76% of As uptake (Table 1) (Xu et al., 2007; Zhao et al., 2010). For Arabidopsis, AsIII efflux was lower than that of tomato and rice, at 25 μg g−1 root or 35–56% of As uptake (Ali et al., 2012; Chen et al., 2013) (Table 1).
Compared to non-hyperaccumulators, AsIII efflux was much lower in *P. vittata* under similar As exposure. In our study after exposing to 10 μM AsV for 28 d, AsIII was extruded into external medium at 0.27 μg g\(^{-1}\) root or 12% of As uptake (Fig. 3C,D). This result agreed with previous studies on *P. vittata*, showing AsIII efflux at 0.28–2.0 μg g\(^{-1}\) root or 4–15% of AsV uptake (Su et al., 2008; Huang et al., 2011) (Table 1). Thus, our results, together with previous studies, clearly showed that *P. vittata* had a lower AsIII efflux when exposing to low As at 5–20 μM AsV than non-hyperaccumulators. The low AsIII efflux is consistent with its highly efficient As detoxification and As accumulation in vivo. This study highlighted the first evidence of AsIII efflux by *P. vittata* after exposure to high AsV, however, the underlying mechanisms are unclear and warrant further study.

### Acknowledgments

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### References


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**Table 1**

Comparison of AsIII efflux activities in different plant species.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>AsV (μM)</th>
<th>Time (d)</th>
<th>AsIII efflux (μg g(^{-1}) root FW)</th>
<th>AsIII efflux (% of AsV uptake)</th>
<th>Phosphate Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>10</td>
<td>1</td>
<td>61</td>
<td>64</td>
<td>+P</td>
<td>Xu et al., 2007</td>
</tr>
<tr>
<td>Rice</td>
<td>10</td>
<td>1</td>
<td>67</td>
<td>69</td>
<td>+P</td>
<td>Su et al., 2008</td>
</tr>
<tr>
<td>Rice</td>
<td>5</td>
<td>1</td>
<td>9</td>
<td>18</td>
<td>+P</td>
<td>Zhao et al., 2010</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>10</td>
<td>1</td>
<td>25</td>
<td>35</td>
<td>+P</td>
<td>Duan et al., 2012</td>
</tr>
<tr>
<td>Wheat</td>
<td>10</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>+P</td>
<td>Ali et al., 2012</td>
</tr>
<tr>
<td>Pteris vittata</td>
<td>5</td>
<td>1</td>
<td>0.28</td>
<td>5</td>
<td>+P</td>
<td>Chen et al., 2013</td>
</tr>
<tr>
<td>Pteris vittata</td>
<td>20</td>
<td>1</td>
<td>1.5</td>
<td>15</td>
<td>+P</td>
<td>Shi et al., 2015</td>
</tr>
<tr>
<td>Pteris vittata</td>
<td>10</td>
<td>28</td>
<td>0.22(^a)</td>
<td>12</td>
<td>+P</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\) All plants were wild type as no mutants or transgenic plants were included.

\(^b\) These data were normalized by As exposure days.


