Comparison of root absorption, translocation and tolerance of arsenic in the hyperaccumulator *Pteris vittata* and the nonhyperaccumulator *Pteris tremula*

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Summary

• Several fern species can hyperaccumulate arsenic, although the mechanisms are not fully understood. Here we investigate the roles of root absorption, translocation and tolerance in As hyperaccumulation by comparing the hyperaccumulator *Pteris vittata* and the nonhyperaccumulator *Pteris tremula*.

• The two species were grown in a pot experiment with 0–500 mg As kg^{-1} added as arsenate, and in a short-term (8 h) uptake experiment with 5 μM arsenate under phosphorus-sufficient conditions.

• In the pot experiment, *P. vittata* accumulated up to 2500 mg As kg^{-1} frond d. wt and suffered no phytotoxicity. *P. tremula* accumulated < 100 mg As kg^{-1} frond d. wt and suffered severe phytotoxicity with additions of ≥ 25 mg As kg^{-1}. In the short-term uptake experiment, *P. vittata* had a 2.2-fold higher rate of arsenate uptake than *P. tremula*, and distributed more As taken up to the fronds (76%) than did *P. tremula* (9%).

• Our results show that enhanced root uptake, efficient root-to-shoot translocation, and a much elevated tolerance through internal detoxification all contribute to As hyperaccumulation in *P. vittata*.

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

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Introduction

Arsenic is highly phytotoxic, with toxicity threshold concentrations in plant tissues normally varying from 5 to 100 mg kg^{-1} d. wt (Kabata-Pendias & Pendias, 1992). However, some plant species are able to grow on As-contaminated soils, because of either low bioavailability of As and /or increased resistance by plants. A common mechanism of As resistance is decreased uptake of arsenate as a result of the suppression of the high-affinity phosphate-uptake systems (Meharg & Hartley-Whitaker, 2002). Furthermore, most plant species transport a very limited amount of As from roots to shoots, resulting in a small shoot-to-root As concentration ratio, for example < 0.02 in tomato (*Lycopersicon esculentum*; Burló *et al.*, 1999); < 0.1 in *Brassica juncea* (Pickering *et al.*, 2000); and < 0.2 in rice (*Oryza sativa*; Marin *et al.*, 1992). For the above reasons, As is not usually accumulated to high concentrations in plant shoots.

Accumulation of > 1000 mg As kg^{-1} d. wt in shoots, without symptoms of phytotoxicity, is unusual in terrestrial plants and can be considered as hyperaccumulation. Other common traits associated with metal-hyperaccumulating plants include a bioaccumulation factor (the ratio of metal concentration in shoots to that in soil) of > 1, and a shoot-to-root concentration ratio of > 1 (McGrath & Zhao, 2003). The first As hyperaccumulator described recently was the fern *Pteris vittata*, which accumulated up to 7500 mg As kg^{-1} in its fronds at an As-contaminated site containing 18–1600 mg kg^{-1} total As in the soil, and up to 22 630 mg As kg^{-1} in the fronds in a pot experiment when 1500 mg As kg^{-1} was added to a soil (Ma *et al.*, 2001). The As bioaccumulation factor was > 10. *P. vittata* is also highly tolerant to As, with a threshold

concentration for phytotoxicity of between 7000 and 10 000 mg As kg^{-1} in frond d. wt (Lombi *et al.*, 2002; Tu & Ma, 2002; Wang *et al.*, 2002).

In addition to *P. vittata*, several other As-hyperaccumulating fern species belonging to the order Pteridales have recently been identified, including *Pteris cretica*, *Pteris longifolia* and *Pteris umbrosa* (Zhao *et al.*, 2002) and *Pityrogramma calamelanos* (Visoottiviseth *et al.*, 2002). Interestingly, not all species within the *Pteris* genus are As hyperaccumulators. Meharg (2003) reported that *Pteris straminea* (probably *Pteris dentata* Straminea) and *Pteris tremula* did not hyperaccumulate As in their fronds. It is not clear from the study of Meharg (2003) whether the lack of As-hyperaccumulating ability in *P. dentata* and *P. tremula* is caused by a lack of internal tolerance, a lower rate of As uptake, and/or lower root-to-frond transport of As compared with other As hyperaccumulators. Comparison of the As-hyperaccumulating and nonhyperaccumulating *Pteris* species should provide further insight into the mechanisms of As hyperaccumulation. Similar comparative physiological studies have been carried out on other metal hyperaccumulators. For example, Krämer *et al.* (1997) showed that the nickel hyperaccumulator *Thlaspi goesingense* and the nonhyperaccumulator *Thlaspi arvense* took up Ni at a similar rate under nontoxic conditions, and that internal Ni tolerance alone is sufficient to explain the Ni hyperaccumulator phenotype observed in the former when compared with the latter species. By contrast, zinc hyperaccumulation in *Thlaspi caerulescens* involves a much larger uptake rate, as well as greater root-to-shoot transport, when compared with the nonhyperaccumulator *T. arvense* (Lasat *et al.*, 1996).

In the present study we compared As uptake and tolerance in the As hyperaccumulator *P. vittata* and the nonhyperaccumulator *P. tremula*. Our objective was to evaluate the roles in As hyperaccumulation of root absorption, root-to-shoot translocation and tolerance.

Materials and Methods

Pot experiment

A pot experiment was carried out to compare two *Pteris* species, *P. vittata* and *P. tremula*, in terms of As accumulation and tolerance. Spores of *P. vittata* and *P. tremula* were germinated on a general-purpose compost. At the three- to four-frond stage sporelings were transferred to plastic pots, each containing 1 kg air-dried compost. One plant was grown in each pot. The compost was amended with arsenate (Na_2HAsO_4) at concentrations of 0, 12.5, 25, 50, 100, 250 and 500 mg As kg^{-1}. Each treatment was replicated four times. Pots were arranged randomly on a bench inside a glasshouse with day/night temperatures of 25/20°C (16/8 h), and a minimum light intensity of 350 μmol m^{-2} s^{-1}. Fronds were harvested on day 35 after transplanting. In some of the treatments with As additions, some of the frond tissues of *P. tremula* died during the experiment. Dead tissues were excluded from biomass determination and chemical analysis. Plant samples were washed thoroughly with deionized water, and dried at 60°C for 24 h.

Additional pots without plants were set up for the extraction of pore water samples from the compost. The As treatments were the same as described above, and each was replicated four times. Pots were covered with black plastic sheeting and placed inside the glasshouse. A porous plastic soil-moisture sampler (Eijekelkamp, Agrisearch Equipment, the Netherlands) was inserted into the middle of the compost inside each pot. Pore water was extracted weekly after the addition of As for 4 wk. Each extraction was done at 16 h after the compost had been watered to 80% of its water-holding capacity (Knight *et al.*, 1998).

Short-term arsenate-uptake experiment

A hydroponic experiment was carried out to compare the rate of arsenate uptake by *P. vittata* and *P. tremula*. Plants of the two species were transferred to hydroponic culture at the four-frond stage. Plant roots were washed carefully with deionized water to remove adhering compost, and transferred to 350 ml pots (one plant per pot) containing a nutrient solution which was a modified Hoagland solution with half-strength major nutrients and full-strength micronutrients (except that iron was supplied as Fe-EDDHA (ethylenediamine-di(o-hydroxyphenylacetic acid) at 100 μM) (Hewitt, 1966). The nutrient solution was aerated continuously and renewed weekly. The experiment was conducted inside a controlled-environment growth chamber with the following conditions: 16 h light period with a light intensity of 350 μmole m^{-2} s^{-1}, 25/20°C day/night temperatures, and 70% relative humidity. After 3 wk preculture, roots of intact plants were rinsed with deionized water and transferred to a pretreatment solution containing 0.5 mM $CaCl_2$ and 5 mM MES (2-morpholinoethanesulphonic acid) with pH adjusted to 6.0. Eight replicate plants were included for each species. After 12 h, when the light period had already started for 2 h, the pretreatment solution was replaced with 320 ml uptake solution containing 5 μM arsenate (Na_2HAsO_4) together with 0.5 mM $CaCl_2$ and 5 mM MES with pH adjusted to 6.0. The uptake solution was aerated vigorously and continuously. At 0, 15 and 30 min and thereafter every 30 min at intervals up to 8 h, 0.3 ml uptake solution was removed for determination of As concentration, and replaced with 0.3 ml deionized water. Water losses through transpiration were compensated by addition of deionized water at hourly intervals. The temperature during the uptake experiment was maintained at 25 ± 0.5°C. After 8 h, roots were separated from shoots, rinsed with deionized water, blotted dry and weighed. Plant materials were dried at 60°C for 24 h.

Chemical analysis

Concentrations of As in the pore water and uptake solution samples were determined using atomic absorption spectroscopy

(4100ZL, Perkin-Elmer, Wellesley, MA, USA) equipped with a flow-injection hydride-generation unit (FIAS 400, Perkin-Elmer) following a prereduction step using KI and ascorbic acid. The instrument has a detection limit for As in solution of 0.1 μg l^{-1}, equivalent to 0.05 μM As in the uptake solution after dilution. Dried plant samples were ground to < 0.5 mm and digested with a mixture of HNO_3 and $HClO_4$ (85/15 v/v). Arsenic and other elements in the digests 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.

Statistics

ANOVA was performed using GENSTAT 5 (Numerical Algorithms Group, 1998).

Results

Pot experiment

Arsenic concentrations in pore water increased with the amount of As added to the compost, varying from 19 μM in the 12.5 mg As kg^{-1} treatment to 126 μM in the 500 mg As kg^{-1} treatment in the samples extracted 1 wk after As addition. Pore water As decreased linearly with incubation time (Fig. 1), and by week 4 after addition the concentrations had decreased by 12–41%.

In the absence of As addition, *P. tremula* produced 73% larger frond biomass than *P. vittata* (Fig. 2). However, frond biomass of *P. tremula* was affected markedly by the additions of As, with 77–92% reduction in the treatments of 12.5–50 mg As kg^{-1}. Only one out of the four replicate plants survived the 100 mg As kg^{-1} treatment, and no plants survived in the 250 and 500 mg As kg^{-1} treatments. Phytotoxicity symptoms, including leaf chlorosis and necrosis around the edges of the pinnae, appeared in the treatments with As additions of 25 mg kg^{-1} or more. By contrast, frond biomass of *P. vittata* was not significantly affected by the additions of As in any of the treatments (Fig. 2). No phytotoxicity symptoms were observed in *P. vittata*.

Frond As concentration in *P. vittata* increased linearly with the increasing amount of As added to the growth medium, reaching 2500 mg kg^{-1} d. wt in the 500 mg As kg^{-1} treatment (Fig. 3a). By contrast, frond As concentrations in *P. tremula* were < 100 mg kg^{-1} d. wt in all treatments that did not kill the plants. In *P. tremula*, frond As concentration was similar only to that in *P. vittata* with the lowest dose of As addition (12.5 mg As kg^{-1}). Above this level of As addition,

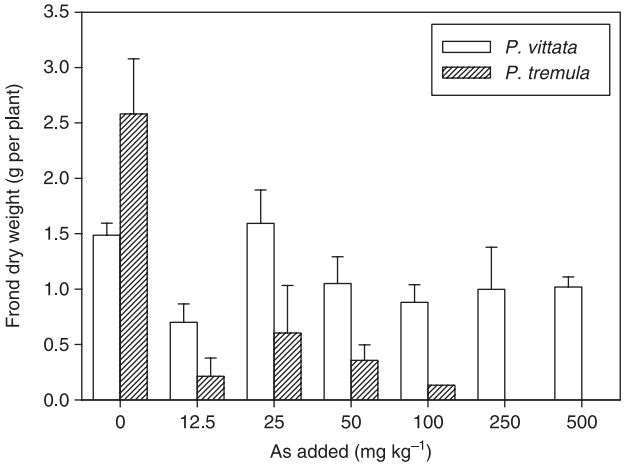

Fig. 2 Effect of arsenic additions on frond dry weights of *Pteris vittata* and *Pteris tremula* in the pot experiment. Results are means + SE (n = 4, except that only one *P. tremula* plant survived in the 100 mg As kg^{-1} treatment).

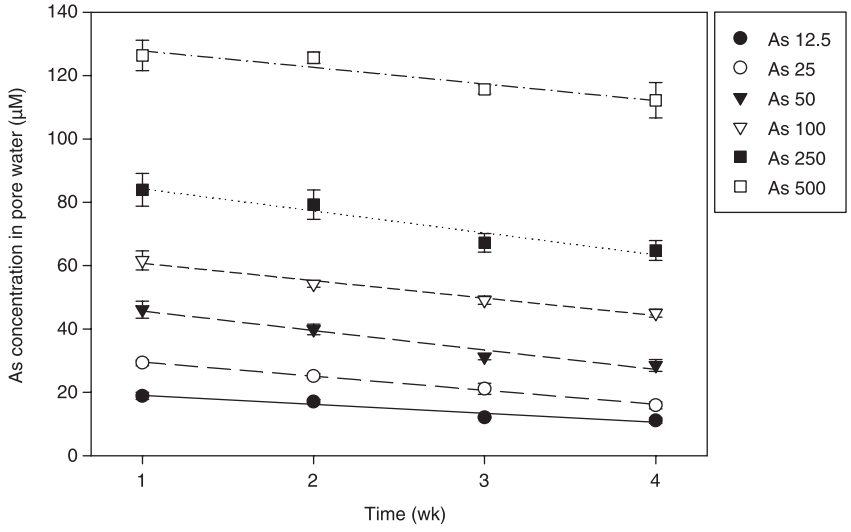

Fig. 1 Arsenic concentration in the pore waters extracted from bare pots amended with different levels of arsenate. Bars, ± SE (n = 4).

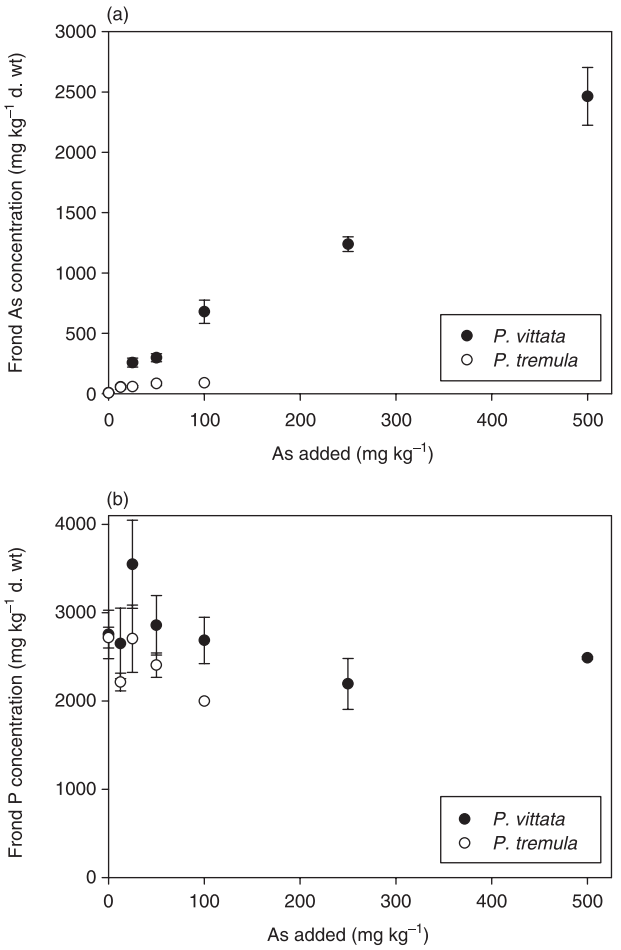

Fig. 3 Effect of arsenic additions on concentrations of (a) As and (b) phosphorus in fronds of *Pteris vittata* and *Pteris tremula* in the pot experiment. Results are means ± SE (n = 4, except that only one *P. tremula* plant survived in the 100 mg As kg^{-1} treatment).

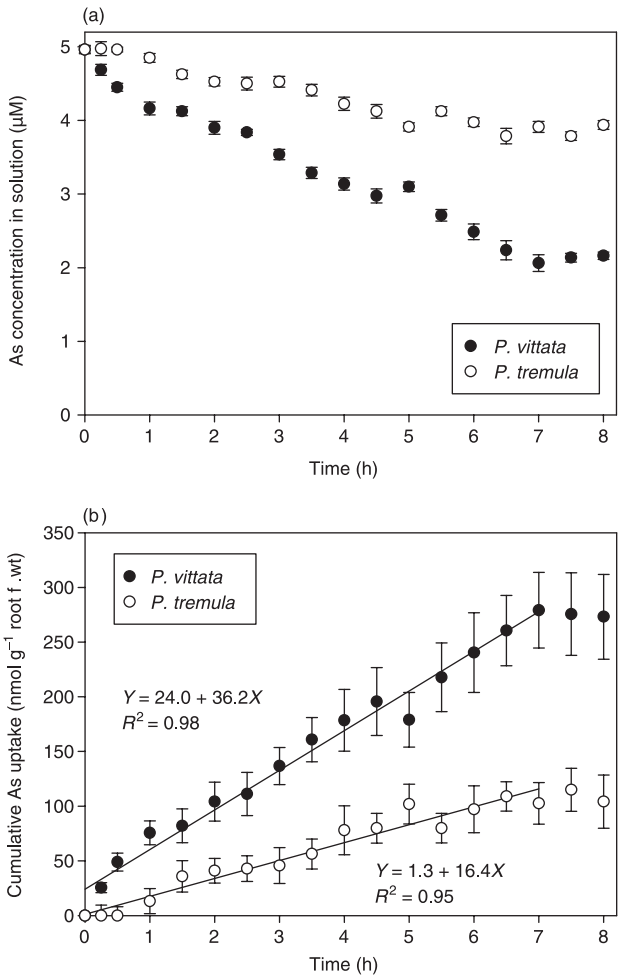

Fig. 4 (a) Depletion curves of arsenate (μM) in the uptake solution by *Pteris vittata* and *Pteris tremula*, and (b) cumulative uptake of arsenate by *P. vittata* and *P. tremula*, expressed on a fresh root weight basis. Results are means ± SE (n = 8).

however, *P. tremula* accumulated much less As in the fronds than did *P. vittata*. Also, the increase of frond As concentration in *P. tremula* was not significant beyond the first dose of As addition.

In the 0–50 mg As kg^{-1} treatments, the average P concentration in the fronds of *P. vittata* was 15% higher than that of *P. tremula* (P < 0.05; Fig. 3b). Frond P concentrations in both species were not significantly affected by the As treatments.

Short-term arsenate-uptake experiment

Depletion of arsenate in the uptake solution was monitored over 8 h, which reflects the net uptake of As by roots. Average root fresh weights were comparable in *P. tremula* (3.68 ± 0.51 g) and *P. vittata* (3.73 ± 0.49 g), whereas frond fresh weight was higher for *P. tremula* (4.2 ± 1.1 g) than for *P. vittata* (2.8 ± 0.5 g). Over the 8 h period, the arsenate concentration in the uptake solution decreased from 5 to 2.2 μM in the presence of *P. vittata*, but only to 3.9 μM in the presence of *P. tremula* (Fig. 4a). Because the rate of depletion also depends on root weight, cumulative As uptake was calculated from the depletion data and expressed on the basis of root fresh weight. The cumulative As uptake was approximately linear in the first 7 h for both plant species (Fig. 4b). During this linear phase, the slope was 36.2 and 16.4 nmol g^{-1} root f. wt h^{-1} for *P. vittata and P. tremula*, respectively, indicating a 2.2-fold higher As accumulation in the former than in the latter.

At the end of the short-term uptake experiment, As concentrations in roots and fronds were determined. In *P. vittata* the As concentrations in fronds were similar to those in the roots (Fig. 5). By contrast, frond As concentration in *P. tremula* was only 3% of that in the roots. On average, 76% of the As taken up by *P. vittata* was distributed to the fronds, whereas in *P. tremula* the percentage was only 9%.

Phosphorus concentrations in fronds and roots were also determined. Frond and root P concentrations of *P. vittata*

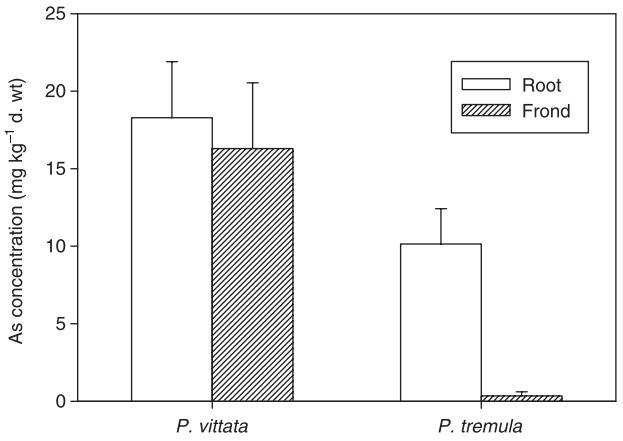

Fig. 5 Arsenic concentration in fronds and roots of *Pteris vittata* and *Pteris tremula* at the end of the depletion experiment. Results are means ± SE (n = 8).

(3280 ± 155 and 12 170 ± 901 mg kg^{-1} d. wt, respectively) were significantly higher than those of *P. tremula* (2544 ± 223 and 7693 ± 982 mg kg^{-1} d. wt, respectively). Both species had a much larger concentration of P in roots than in fronds.

Discussion

In the present study we compared As uptake and tolerance in two closely related fern species, *P. vittata* and *P. tremula*, the former being an As hyperaccumulator (Ma *et al.*, 2001) and the latter a nonhyperaccumulator (Meharg, 2003). In the pot experiment, *P. vittata* accumulated up to 2500 mg As kg^{-1} in the fronds, whereas As concentrations in the fronds of *P. tremula* were smaller than 100 mg kg^{-1}. The two species also differed markedly in As tolerance. *Pteris vittata* was highly tolerant to As, showing no sign of phytotoxicity or reduction in frond biomass with As additions up to 500 mg kg^{-1} (corresponding As concentrations in the pore water were 112–126 μM). By contrast, *P. tremula* was very sensitive to the exposure to As. Frond biomass was decreased markedly at the lowest level of As addition at only 12.5 mg kg^{-1} (corresponding As concentrations in the pore water: 11–19 μM). Furthermore, *P. tremula* showed clear phytotoxic symptoms at As additions of 25 mg kg^{-1} or above, and did not survive the 250 and 500 mg As kg^{-1} treatments. The threshold concentration of As phytotoxicity in *P. tremula* (< 100 mg As kg^{-1} frond d. wt) is similar to the threshold values of 5–100 mg As kg^{-1} d. wt reported in a range of nonaccumulator plants (Kabata-Pendias & Pendias, 1992). Clearly, a greatly elevated tolerance allows *P. vittata* to grow on high-As substrates and realize its potential of As hyperaccumulation. On the contrary, a lack of As tolerance in *P. tremula* prevents it from growing on the substrate with even moderate amounts of As. Arsenic phytotoxicity possibly also led to a depression of As accumulation in *P. tremula*, because frond As accumulation in this species did not increase beyond the first dose of As addition (Fig. 3). Therefore As tolerance can be regarded as one of the important reasons for (or, more precisely, a prerequisite of) As hyperaccumulation in *P. vittata*.

Given that As hyperaccumulators such as *P. vittata* can accumulate and tolerate As up to the percent level in frond dry matter (Tu & Ma, 2002; Wang *et al.*, 2002), the main mechanism of As tolerance must be through internal detoxification. This strategy is clearly different from the exclusion mechanism employed by many tolerant plant species growing on As-contaminated sites, which suppress As uptake through a suppression of high-affinity phosphate-transport pathway (Meharg & Macnair, 1991, 1992; Bleeker *et al.*, 2003). In *P. vittata* internal detoxification probably involves a reduction of arsenate to arsenite (Wang *et al.*, 2002; Zhang *et al.*, 2002; Webb *et al.*, 2003) and a subsequent sequestration in the vacuoles (Lombi *et al.*, 2002). Phytochelatins, which have been shown to play a key role in both constitutive and adaptive tolerance to As in nonhyperaccumulating plant species (Ha *et al.*, 1999; Hartley-Whitaker *et al.*, 2001; Schat *et al.*, 2002; Bleeker *et al.*, 2003), appear to have a limited role in As tolerance in *P. vittata* (Zhao *et al.*, 2003). A recent study showed that < 1% of the As in fronds of the hyperaccumulator *P. cretica* was complexed with phytochelatins (Raab *et al.*, 2004).

Is a strong internal detoxification mechanism alone sufficient to explain the phenomenon of As hyperaccumulation in *P. vittata*, as has been suggested for Ni hyperaccumulation in the hyperaccumulator *Thlaspi goegingense* (Krämer *et al.*, 1997)? Results from the pot experiment appear to support this hypothesis, because frond As concentrations were similar between *P. vittata* and *P. tremula* (≈ 55 mg As kg^{-1}) in the lowest As treatment (Fig. 3). However, in this treatment frond biomass of *P. tremula* was already significantly smaller than that in the control treatment, and also smaller than that of *P. vittata* in the same treatment (Fig. 2). Consequently, the total amount of As accumulated in the fronds was 3.4-fold higher in *P. vittata* than in *P. tremula*.

To address whether *P. vittata* possesses an enhanced ability of As uptake compared with *P. tremula* in addition to an enhanced internal tolerance, we compared arsenate uptake by the two species in the short term (8 h) and at a relatively low arsenate concentration (5 μM). This experiment was designed to avoid potential side-effects of As toxicity on root uptake. During the linear uptake phase, arsenate uptake was 2.2-fold faster in *P. vittata* than in *P. tremula*. The difference was already noticeable in the first 30 min of uptake (Fig. 4). Similarly, a recent study by Huang *et al.* (2004), which evaluated the potential of As-hyperaccumulating ferns for removing As from drinking water, showed that *P. vittata* was much more efficient in arsenate uptake than the nonhyperaccumulating fern species *Nephrolepis exaltata*. In our study the kinetic parameters for arsenate influx could not be calculated from

the As depletion data in this experiment, because a near-complete depletion in the uptake solution is required for an accurate calculation (Claassen & Barber, 1974), which was not achieved for both species. Nevertheless, the large difference between the two species observed during the initial phase of the depletion experiment indicates a large difference in their maximum influx velocity (V_{max}), which suggests a higher density of transporters for arsenate uptake on the plasma membranes of root cells in *P. vittata* than in *P. tremula*. Similarly, it has been shown that the Zn hyperaccumulator *T. caerulescens* has a fourfold higher V_{max} for Zn influx than the nonaccumulator *T. arvense* (Lasat *et al.* (1996). The affinity (K_m) of the transporters to arsenate could not be ascertained in the present study. It should be pointed out that a 2.2-fold difference in arsenate influx rate, observed under hydroponic conditions, would make a significant difference to As uptake only when plants are grown on soils with a high As supply (e.g. in all +As treatments of the pot experiment, which contained much higher concentrations of As in the pore waters than the initial As concentration used in the hydroponic experiment). In soils with a low As supply, root uptake of arsenate, like phosphate, is likely to be limited by the diffusion process of the ions to the root surface. Using a nutrient uptake-simulation model, Silberbush & Barber (1983) showed that the predicted P uptake by soybean growing on a soil with a relatively low P status was more sensitive to changes in soil P supply than to root uptake kinetics.

Arsenate is a chemical analogue of phosphate, and there is ample evidence that plants take up arsenate via the phosphate-transport systems (Meharg & Hartley-Whitaker, 2002). We found that phosphate competed with arsenate uptake in *P. vittata*, whereas P starvation enhanced arsenate uptake (Wang *et al.*, 2002). In both pot and hydroponic experiments of the present study, both plant species were supplied with sufficient levels of P, which is expected to decrease arsenate influx. Whether the two *Pteris* species respond differently to P starvation in terms of arsenate uptake kinetics remains unknown.

Apart from an enhanced root uptake, *P. vittata* also transported As from roots to fronds much more efficiently than *P. tremula*. This difference is clearly demonstrated by the distribution of As at the end of the short-term uptake experiment (Fig. 5), with 76 and 9% of the As taken up being distributed to the fronds in *P. vittata* and *P. tremula*, respectively. Efficient root-to-shoot translocation is a typical character of metal hyperaccumulators, although the mechanisms responsible for this trait are still poorly understood (McGrath & Zhao, 2003). Possible explanations include decreased vacuolar sequestration in the root cells, or an enhanced xylem loading.

In conclusion, our results show that *P. vittata* possesses three common traits associated with metal/metalloid hyperaccumulators: enhanced root uptake; efficient root-to-shoot translocation; and a greatly elevated tolerance through internal detoxification. All three traits contribute to the phenotype of As hyperaccumulation in *P. vittata* when compared with the nonaccumulator *P. tremula*.

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