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TRANSPORT OF ARSENITE BY THE ARSENIC HYPERACCUMULATING BRAKE FERN *PTERIS VITTATA* IS INHIBITED BY MONOVALENT SILVER

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SUMMARY

The capacity of silver (Ag^+) to inhibit the transport of arsenite by the arsenic (As) hyperaccumulating fern *Pteris vittata* was investigated. The hydroponic growth medium was supplemented with 200 µM arsenite and 1, 10, or 100 µM Ag^+, with roots and pinnae harvested intermittently following treatment. Treatment with Ag^+ significantly reduced transpiration and prevented the hyperaccumulation of As. A study of competitive uptake between arsenite and its analog antimonite showed that arsenite significantly reduced the concentration of antimonite in *P. vittata*, but antimonite had no effect on As accumulation. The results collectively suggest that uptake of arsenite by *Pteris vittata* is mediated by membrane transporters sensitive to Ag^+ inhibition that may also mediate antimonite transport, such as members of the major intrinsic protein family.

Key words*: Arsenic, arsenite, hyperaccumulation, phytoremediation, *Pteris vittata

INTRODUCTION

Arsenic (As) is a highly toxic metalloid that commonly occurs in soils and waters as arsenate or arsenite. Arsenate is the predominant form under aerobic conditions, whereas, anaerobic and reducing conditions favor the formation of arsenite. From an agricultural perspective, arsenite is a major contaminant of irrigation waters, originating from natural as well as anthropogenic sources. Arsenite is taken-up by various plants and its accumulation in rice is a serious concern, given the wide reliance on this crop as a staple food (Meharg and Jardine 2003). However, the mechanism utilized by higher plants in the uptake of arsenite, has not been conclusively established

Arsenite is taken up by *Saccharomyces cerevisiae* and *Escherichia coli* via members of the major intrinsic protein (MIP) family of transporters (Wysocki *et al*. 2001). This family consists of a group of multifunctional channels that mediate the transport of uncharged and neutral solutes (e.g., glycerol and urea) (Chrispeels and Maurel 1994, Javot and Maurel 2002, Maurel 1997). For bacteria and yeast, antimonite and arsenite have been shown to compete for the same transporter (Meng *et al*. 2004). Similarly, a competition study with rice showed that antimonite uptake is inhibited by arsenite in a dose dependent fashion, suggesting a possible role of these transporters in arsenite transport by plants. An attempt was made to use Hg^+ as a channel blocker with whole plants, but the results were ambiguous due to the presumed cytotoxicity of this element (Meharg and Jardine 2003).

The objective of this study was to examine the possible role of plant root MIPs in the uptake of arsenite

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using Ag^+ as the channel blocker. This cation is less toxic than mercurial compounds and has a similar effect on transport (Montalvetti *et al*. 2004). For example, Ag^+ reportedly reduced the water permeability of beet root plasma membrane by almost 74% (Niemietz and Tyerman 2002). Furthermore, Ag^+ has been reported to be a more potent blocker than Hg^{2+} in reducing the water permeability through the Nodulin-26 like intrinsic protein (Dean *et al*. 1999). Presumably, this effect also extends to solutes transported into roots via these channels. For this study, the Chinese brake fern (*Pteris vittata*) was used as the model system. *Pteris vittata* is known for the ability to hyperaccumulate arsenate or arsenite to >10,000 mg kg^{-1} dw in the above ground biomass (Ma *et al*. 2001). This characteristic of brake fern could be exploited for As phytoremediation, provided sufficient information is available on As transport and metabolism. A second goal of this research was to determine if arsenite transport by this hyperaccumulator occurs by a mechanism similar to that reported for rice.

MATERIALS AND METHODS

Plant materials and growth conditions

Two-month old Chinese brake (*P. vittata*) fern plants, propagated from spores (Poynton *et al*. 2004), were purchased commercially (Edenspace Systems, Inc., Chantilly, VA, USA) and transferred from a potting mixture to nutrient solution with constant aeration in 2 L black polyethylene pots. The nutrient solution was changed weekly. The composition of the nutrient solution was 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 µM KCl, 12.5 µM H_3BO_3, 1 µM $MnSO_4$, 1 µM $ZnSO_4$, 0.4 µM $CuSO_4$, 0.1 µM Na_2MoO_4, 0.1 µM $NiSO_4$. The pH was adjusted to 5.5 with 1 mM MES (2-[N-morpholino]ethanesulfonic acid) titrated with KOH and iron was supplied as 5 µM Fe-EDDHA (N,N'-ethylenediamine-di (O-hydroxy-phenylacetic acid). The dilute nutrient solution mentioned below was a 20-fold dilution of this nutrient solution. Plants were grown under a 12 h photoperiod; at ~ 300 µmol photons $m^{-2}s^{-1}$, 25° /20° ± 2 °C day/night temperatures, and relative humidity of approximately 60%.

Arsenite transport experiments

After three weeks of acclimation in hydroponics, the plants were transferred into 250 ml Erlenmeyer flasks with 200 ml of dilute strength nutrient solution for one week. The flasks were wrapped with aluminum foil to exclude light and the solution was aerated. Twenty-four hours prior to As treatment, ferns were removed, the roots were washed with deionized water, and the plants transferred to 200 ml of aerated pretreatment medium (0.5 mM $CaCl_2$ and 5 mM MES with pH adjusted to 5.5 with KOH) in similar flasks. This pretreatment was performed to desorb the root cell wall space of residual nutrients from the solution. There were no noticeable signs of nutrient deficiency observed in these ferns during any of the preconditioning periods. Water lost by transpiration was replaced as needed. After pretreatment, ferns were used in two arsenite transport experiments. For each experiment, four replicates per treatment were used in a randomized block design.

Inhibition of arsenite uptake by Ag^+: Twelve hours prior to As treatment, the roots of *P. vittata* at the four-frond stage were rinsed with deionized water and transferred to dilute nutrient solution containing Ag^+ (as $AgNO_3$) at 0, 1, 10, or 100 µM. After 12 h of Ag^+ treatment, arsenite was introduced to a final concentration of 200 µM. Buffering the pH of the uptake solution to 5.5 prevented the precipitation of Ag^+ with arsenite. The ferns were exposed to arsenite for 15 days. Transpiration was monitored daily after the treatment and water lost to transpiration was replaced in the uptake solution by addition of deionized water. At five-day-intervals after arsenite treatment, three to four pinnae from each of the treated plant were excised for elemental analysis. The remaining plant tissues were harvested for As and Ag^+ analysis at the end of the experiment.

Competition between arsenite and antimonite for uptake: Following a one week preconditioning, *P. vittata* plants were transferred to fresh uptake solutions containing 0.5 mM $CaCl_2$ + 5 mM MES (pH 5.5 adjusted with KOH) supplemented with 100 µM arsenite, 100 µM antimonite, or 100 µM arsenite + 100 µM antimonite. The treatments lasted for 8 h after which the

roots and the shoots were harvested separately for elemental analysis.

Elemental analysis

Harvested plant materials were rinsed three times with deionized water and oven dried at 60º C. Tissues were ground to a fine powder and digested with trace metal grade HNO_3 and 30% H_2O_2 after EPA Method 3050b. Total As, Ag and Sb were determined by graphite furnace atomic absorption spectrometry (GFAAS) using a Varian 220FS AAS (Walnut Creek, CA).

Data analyses

Statistical analyses were performed using SPSS for Windows ver. 12 (SPSS, Inc.) for repeated measures analysis of variance (ANOVA) of the elemental data from the three experiments and regression analysis of the transpiration data.

RESULTS AND DISCUSSION

Channel blocker studies have used specific and non-specific inhibitors which reduce transpiration by exerting restrictions to water uptake and block the movement of small neutral solvents into the roots of plants. Both mercurial and silver compounds have been reported as potent broad spectrum MIP inhibitors in plant and animal cell systems. Here, 10 and 100 μM Ag^+ treatments resulted in a significant decrease in both evapotranspiration and As accumulation over 15 days of exposure in *P. vittata*. These treatments reduced As accumulation in the roots ($p < 0.05$, Fig. 1A) and shoots ($p < 0.05$, Fig. 1B) canceling the hyperaccumulating ability of this ferns. In the absence of Ag^+ arsenite hyperaccumulated to concentrations that have been observed previously. Nonlinear regression revealed that in the absence of Ag^+, there was a significant relationship between the overall As accumulated and water transpired ($R^2 = 0.786$, $p < 0.05$) (Fig. 1C) as well as consistent evapotranspiration time (Fig 1D). When the brake ferns were exposed to arsenite in the presence of 10 or 100 mM Ag^+, evapotranspiration occurred at a significantly reduced rate ($p < 0.05$). The concentration-dependent effect of 10 μM or higher Ag^+ on transpiration

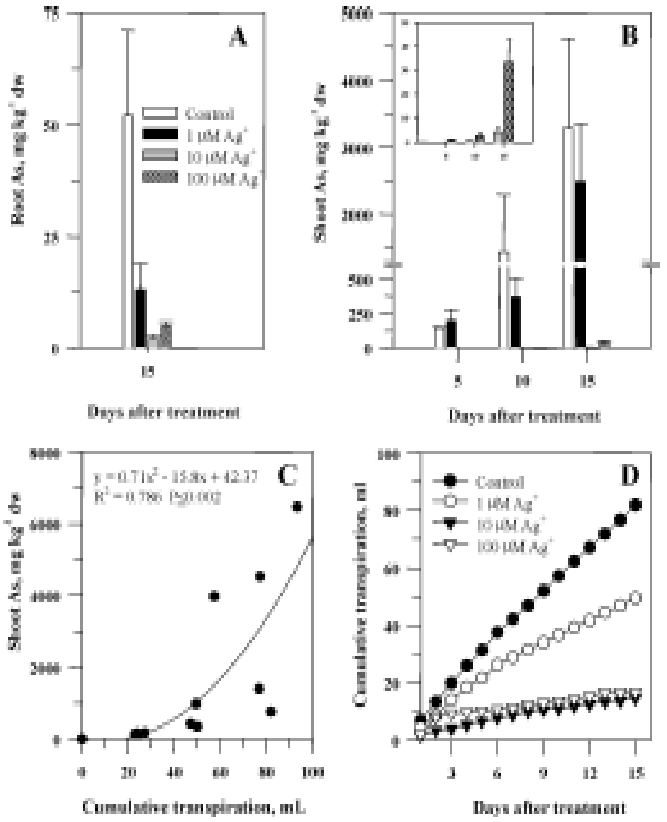

Fig. 1. The effect of Ag^+ inhibition on As accumulation in root and transpiration by the As-hyperaccumulating brake fern *Pteris vittata* L. Error bars represent the standard error of the mean (*n* = 4). For some data points, the standard error is smaller than the size of the symbol. (A) Total As concentration in roots at the end of the 15 d Ag^+ exposure period. (B) Total As concentration in shoots (pinnae and rachis) 5, 10, and 15 days after Ag^+ treatment. Legend as in Fig. 1A. Inset: The effect of 10 μM and 100 μM Ag^+ treatments on the As concentration of shoots. Axes on the inset correspond to the main axes of Fig. 1B. (C) Nonlinear regression of the relationship between total As accumulation and transpiration for brake ferns exposed to arsenite in the absence of glyceroaquaporin inhibitors, showing the dependence of arsenite accumulation on transpiration. (D) Cumulative water loss due to transpiration for *P. vittata* plants treated with Ag^+ over a 15 d time course.

was similar in magnitude to that observed for studies with soybean and beet plasma membrane vesicle shrinking rate (Niemietz and Tyerman 2002). One interpretation of the results is that Ag^+ as an MIP blocker reduced both water and arsenite transport into the fern roots. As this was accompanied by a decrease in transpiration of ~90%, the magnitude of the decreases could indicate a

relationship. Similarly, the 1 μM silver treatment did not significantly affect As accumulation but had only a modest effect on transpiration, perhaps because the concentration may simply fall below the threshold for a significant effect on As transport.

An alternate interpretation could be that silver in the media created a stress which caused the fern to close stomata, reducing evapotranspiration and the translocation of arsenite to shoots. A similar conclusion, citing possible cytotoxocity, was put forth to explain the inhibition of arsenite transport by rice treated with Hg^+ (Meharg and Jardine 2003). Several factors argue against the possibility of Ag^+ stress or toxicity here. There were no signs of physiological stress in ferns exposed to any concentration of Ag^+. There was no significant difference in biomass between the different Ag^+ treatments during the experiment. Silver was restricted mainly to the roots (~ 1.2 mg kg^{-1} dw) and poorly translocated to shoots (<0.02 mg kg^{-1} dw). Phytotoxicity of Ag^+ varies but is typically observed at concentrations >700 μM (Ratte 1999). Direct effects of Ag^+ on stomatal and cuticular water conductance are possible, but these occur when Ag^+ precipitates out (Schreiber *et al*. 2006). Given that there was little Ag^+ present in the fronds of the Chinese brake ferns, this effect of Ag^+ seems unlikely. In the absence of evidence of Ag^+-induced stress, the blocking of arsenite transport via MIPs is plausible but would require additional study to verify. Blocking of MIPs would likely lead to collateral effects on other membrane proteins and transporters as well as membrane integrity and cell water relations. As no other measures of root membrane integrity or transport capacity were made in this study, an indirect effect Ag^+ on arsenite transport is possible. However, the results here, showing such a dramatic inhibition of arsenite accumulation by a hyperaccumulator in the absence of overt symptoms of stress, toxicity, or Ag^+ uptake, suggest a potentially important physiological mechanism. The results do indicate that arsenite transport is transpiration-dependent (Fig. 1C) and is responsive to Ag^+ in much the same way that arsenite transport by rice is responsive to Hg^+. This implies that despite its status as an As hyperaccumulator, the mechanism of arsenite transport by *P. vittata* is similar to that of normal plants such as rice.

This conclusion is somewhat supported by the results of the As-Sb competition study. Both As and Sb form oxyanions in water and display similar chemical behaviors in the environment. The prokaryote glycerol facilitator, GlpF from *E. coli* and Fps1p from eukaryote *S. cerevisiae* are reportedly permeable to arsenite and antimonite (Meng *et al*. 2004). Reportedly arsenite uptake in plants is affected by antimonite in a reciprocally dose dependent manner, similar to yeast. In this study, the accumulation of antimonite was decreased by almost 50% by arsenite treatment, but curiously there was not a reciprocal effect of antimonite on arsenite accumulation (Fig. 2). The reduction of Sb accumulation in the shoots of the plant when both arsenite and antimonite were supplied in the medium is similar to the findings reported in rice (Meharg and Jardine 2003). While these data imply that arsenite and antimonite are transported on a similar transporter, the lack of an effect of Sb on As accumulation may indicate that there are differences in the specificity of metalloid transport for the fern. These data suggest an interaction between these two elements, but an interaction perhaps more intricate than simple competitive uptake.

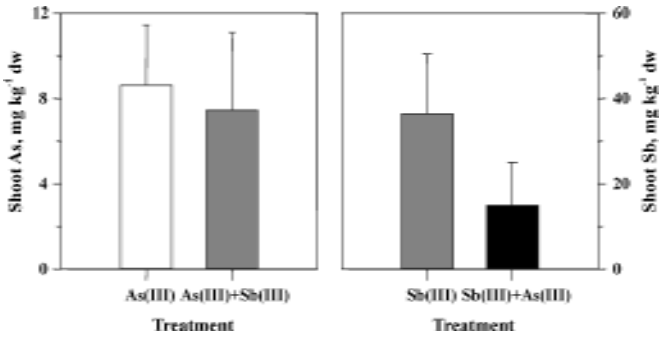

Fig. 2. Influence of arsenite and antimonite interactions on As and Sb accumulation in shoots of *P. vittata*. Treatments used were either arsenite or antimonite alone or combined treatments of arsenite + antimonite. Error bars represent the standard error of the mean (*n* = 4)

Collectively, the results presented here offer some support for the hypothesis that arsenite uptake by the roots of the As hyperaccumulating fern *P. vittata* may be mediated by root MIPs. At the very least, the Ag^+ and Sb treatments indicate that the dependence of arsenate on transpiration and the interaction with antimonite are consistent with the transport mechanism

for arsenite observed in rice (Meharg and Jardine 2003). More conclusive data is clearly needed to fully evaluate this hypothesis and confirm that the results obtained in response to Ag^+ are not simply due to Ag^+ phytotoxicity. As the *Pteris* ferns show considerable promise and flexibility for the phytoremediation of As contamination, a more detailed understanding of the transport mechanisms for different chemical species of As is required. If in fact arsenite is transported on MIPs or related transporters, then the capacity to hyperaccumulate arsenite may be intimately associated with plant water status and evapotranspiration. The manipulation of water relations could therefore, help maximize the capacity of these unique ferns for arsenite phytoremediation.

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