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Impacts of sulfur regulation *in vivo* on arsenic accumulation and tolerance of hyperaccumulator *Pteris vittata*

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ABSTRACT

The impacts of the regulation of sulfur (S) metabolism *in vivo* on arsenic (As) and S species and on As accumulation by *Pteris vittata* L. were investigated using a synchrotron-based X-ray-absorption fine structure method. The S assimilation inhibitor L-buthionine-sulfoximine (BSO) markedly inhibited As reduction, doubling arsenate (As(V)) content in *P. vittata* rhizoids. The resulting As transport blockage in rhizoids, decreased As movement to aboveground tissues by 47%. The significant impact of BSO demonstrated the vital role of sulfhydryl groups (—SH) as reductants in As(V) reduction and confirmed the importance of As(V) reduction in As accumulation in this fern. The S metabolism accelerant *O*-acetyl-L-serine resulted in the appearance of large amounts of As–SH in rhizoids and had no obvious impact on As accumulation, but with As stress conditions, effectively increased plant biomass, possibly through chelation of excess As with —SH. Thus, —SH appeared able to act as both a reductant and a chelator of As in *P. vittata*, and the ratio of —SH to As may have been a factor that determined the specific role of —SH in *P. vittata* under these conditions.

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1. Introduction

Arsenic (As) is a highly toxic metalloid that poses a hazard to microbes, plants, animals, and human beings (Kaise et al., 1985). Removal of As from soil by phytoextraction, using an As hyperaccumulator, has attracted increasing attention owing to its economic efficiency, environmental friendliness, and ease of application (Chen et al., 2002). The mechanism for As uptake by the hyperaccumulating fern *Pteris vittata* L., also known as the Chinese brake fern, and its ability to tolerate high As concentrations comprise an area of active research (Krämer, 2010).

Arsenic is a redox-sensitive element whose toxicity largely depends on its oxidation state and chemical species. Arsenate, or As(V), acts as a phosphate analog and is transported across the plasma membrane *via* phosphate transport systems. Arsenite, or As(III), reacts with sulfhydryl groups (—SH) of enzymes and tissue proteins, inhibiting cellular function, and contributing to cell death (Webb et al., 2003). Huang et al. (2008) have found that, in *P. vittata* rhizoids, As mainly exists as As(V), while As(III) dominates in fronds, and that the As(V) reduction rate was much faster in *P. vittata* than in normal plants, which is considered an important feature for effective As tolerance and accumulation. Study of As species changes relative to sulfur metabolism in *P. vittata* may help elucidate and extend the understanding of hyperaccumulation mechanisms.

Owing to the close relationship between —SH and different As species, —SH has received intense research attention (Vetterlein et al., 2009). Although Zhang et al. (2004) have concluded that phytochelatins (PCs) play a limited role in the As detoxification of a hyperaccumulator because the observed PCs/As ratio is extremely low, Cao et al. (2004) and Singh et al. (2006) believe that —SH is important in As tolerance, particularly with high As exposure (50–200 $mg\,kg^{-1}$).

Differing from the above research, which focused on the role of —SH detoxification in *P. vittata*, Wei et al. (2010) have found that exogenous reduced glutathione (GSH) and histidine greatly aid As accumulation in *P. vittata* (increased ~80%). They also concluded that the cause of this phenomenon is not consistent with experiments *in vitro* by Duan et al. (2005) and Nagarajan and Ebbs (2010). The latter studies indicated that As reduction in *P. vittata* depends on —SH as an electron transferring intermediate for As(V) to As(III) reduction. In contrast, Wei et al. (2010) have found no impact of S addition on As species abundance. In agreement with the above-mentioned study by Duan et al. (2005). Li et al. (2009) proposed that, rather than serving as a chelator in the hypertolerant plant *Adiantum capillus-veneris*, —SH mainly acts as an electron donor in *P. vittata*.

The exact role of —SH in As accumulation by *P. vittata*, whether acting as a detoxifier or helping in hyperaccumulation, has remained unclear. The goal of this study was to explore the roles

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of —SH in *P. vittata* by investigating alterations in the As species and their concentrations under conditions of positive or negative regulation of S assimilation *in vivo*, being moderated by *O*-acetyl-L-serine (OAS) or L-buthionine-sulfoximine (BSO), respectively. The results could provide useful information regarding the related biochemical processes in the As hyperaccumulator *P. vittata*.

2. Materials and methods

2.1. Plant growth and As treatments

P. vittata were cultured in a modified Hoagland nutrient solution containing 7.5×10^{-4} M K_2SO_4, 6.5×10^{-4} M $MgSO_4$, 0.25×10^{-4} M KCl, 0.5×10^{-3} M $Ca(NO_3)_2$, 6.25×10^{-5} M KH_2PO_4, 2.5×10^{-6} M H_3BO_3, 2.5×10^{-7} M $MnSO_4$, 1×10^{-7} M $CuSO_4$, 1×10^{-6} M $ZnSO_4$, 1.25×10^{-9} M $(NH_4)_6MoO_4$, and 2.5×10^{-5} M Fe-EDTA, all of analytical or trace metal grade (Sinopharm Chemical Reagent, Beijing, China), and the pH was adjusted to 6.2 using dilute H_3PO_4 or NaOH. When seedlings were ~10 cm tall, nutrition treatments were arranged as described below and all treatments performed in quadruplicate.

Negative S regulation was imposed using BSO (Hutter et al., 1997; Zhao et al., 2003), with precultured *P. vittata* transplanted and maintained for 1 week in a nutrient solution containing 10 mg L^{-1} As as disodium hydrogen arsenate hydrate ($Na_2HAsO_4 \cdot 7\ H_2O$, DHAH) and 0.5 mM BSO (Advanced Technology & Industrial Co., Hong Kong, China) in the +BSO treatment and no BSO in the control (designated −BSO).

Positive S regulation was imposed using OAS (Urano et al., 2000), with precultured *P. vittata* transplanted and maintained for 2 weeks in nutrient solution containing 10 mg L^{-1} As as DHAH and 0.5 mM OAS (Advanced Technology & Industrial Co.) in the +OAS treatment and no OAS in the control (designated −OAS).

Plants were all maintained in a greenhouse with a 16 h photoperiod, lighting intensity of 300 mE m^{-2} s^{-1} provided by fluorescent and incandescent lamps, a day/night temperature regime of 26/15 °C, 50% average relative humidity, and nutrient solution renewed every 3 d.

At termination, plants were harvested, washed with tap water, and rinsed three times with deionized water. Each plant was divided into leaves/pinnae, stems/petioles, and roots/rhizoids, freeze-dried under vacuum at −50 °C for 48 h, and stored in a −30 °C freezer prior to X-ray absorption spectroscopy (XAS) assessment. Subsequently, all plant samples were digested with a mixture of $HNO_3/HClO_4$ (5/1, v/v) and the As concentration analyzed using an atomic fluorescence spectrometer (AFS-2202, Haiguang Instrument Co., Ltd., Beijing, China). Data statistical analysis was performed using an independent-samples *t*-test, implemented with SPSS 10.0 software (SPSS, Inc., Chicago, USA, Release 17.0).

2.2. XAS measurement and X-ray absorption near edge structure fitting

Immediately prior to XAS measurement, freeze-dried samples were carefully ground to powder and packed in a 3 cm × 0.7 cm sample holder. Aqueous solutions of sodium arsenite and arsenate (analytically pure) were used as reference compounds for inorganic As(III) and As(V), respectively. As(III)-tris-glutathione, synthesized by adding a 10-fold molar excess of reduced L-glutathione (GSH, Alfa Aesar Chemical Co., Tianjin, China) to a solution of sodium arsenite, was used to model As(III) coordinated to three thiols (Pickering et al., 2000). XAS of As were collected in fluorescence mode at an X-ray absorption fine structure (XAFS) station on a Beamline 14W1 of the Shanghai Synchrotron Radiation Facility (Shanghai, China) and the electron storage ring operated at 3.5 GeV.

Table 1
Biomass and As concentration of *P. vittata* with different treatments.

Treatment	Biomass (DW, g)	As concentration (mg kg^{-1})	
		Aboveground parts	Roots
−BSO	1.0 ± 0.1	3985 ± 685	351 ± 55
+BSO	1.1 ± 0.3	2137 ± 196*	578 ± 84*
−OAS	1.1 ± 0.2	3875 ± 607	315 ± 43
+OAS	1.6 ± 0.4*	3512 ± 107	233 ± 68

Values are means ± standard deviation of 4 replicates.
* Significantly different with respect to control for the same condition ($p < 0.05$); DW, dry weight.

GSH, dimethyl sulfoxide, sodium sulfite, and sodium sulfate were used as reference compounds for —SH, sulfoxide, sulfite, and sulfate, respectively (Li et al., 2009). XAS of S were collected at an XAFS station on a Beamline 4B7A of the Beijing Synchrotron Radiation Facility (Beijing, China) and the electron storage ring operated at 2.2 GeV. In processing, the pre-edge background was removed and normalized, and the X-ray absorption near-edge structure (XANES) spectra of As and S were quantitatively analyzed according to Huang et al. (2008) and Huffman et al. (1991), respectively. Small components (<1%) were excluded from the final fits.

3. Results

3.1. Effects of S regulation on biomass and As concentration in P. vittata

The biomass of +BSO treated plants was not significantly different from the control (−BSO treatment) ($p > 0.05$), indicating that negative S regulation did not influence normal physiological activities with 1 week of As exposure (Table 1). In contrast, the biomass of +OAS treatment plants was significantly higher (~60%) than its associated control (−OAS treatment) ($p < 0.05$). With the −OAS treatment and 2 weeks of As exposure, plant growth was slightly inhibited, accompanied by some visible poisoning symptoms on old pinnae and a relatively slower growth rate of young pinnae. In contrast, *P. vittata* with +OAS treatment grew normally.

The +BSO treatment, producing negative regulation of —SH synthesis, decreased the As concentration in aboveground tissues by 46%, while As increased in the roots by 65% (Table 1). The +OAS treatment, producing a positive —SH regulation, showed a slight decrease in As concentrations in both the aboveground tissues and the roots, but the difference was insignificant ($p > 0.05$).

3.2. Effects of BSO imposed negative S regulation on S and As species in P. vittata

Four peaks were identified in the XANES spectra of *P. vittata* (Fig. 1). To simplify interpretation, the 2471 eV peak was defined as the reduced S form or thiol compounds, that at 2478 eV as the oxidized S form or sulfate, and 2473.9 and 2476.9 eV as the intermediate S forms, sulfoxide and sulfite, respectively. The quantitative method of fitting S species is illustrated in Fig. 1.

With the −BSO control conditions, the oxidized S form was the main storage form in these plants, accounting for 89.7, 91.3, and 57.1% of total S in roots, petioles, and pinnae, respectively (Fig. 2), reduced S the second most abundant form, and the intermediate forms the least. The +BSO treatment significantly decreased the oxidized S in roots, petioles, and old and young pinnae by 43, 12, 4, and 52%, respectively. The appearance of high amounts of intermediate S forms and the significant decrease of oxidized S confirmed the role of BSO as a S assimilation inhibitor. Among the various tissues of *P. vittata*, the reduced and intermediate S forms were more abundant in pinnae than in the roots and petioles. In contrast, BSO

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