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## Research article

Characterization of glutathione reductase and catalase in the fronds of two *Pteris* ferns upon arsenic exposureGina M. Kertulis-Tartar<sup>a,1</sup>, Bala Rathinasabapathi<sup>b</sup>, Lena Q. Ma<sup>a,\*</sup><sup>a</sup> Soil and Water Science Department, University of Florida, Gainesville, FL 32611, USA<sup>b</sup> Horticultural Sciences Department, University of Florida, Gainesville, FL 32611, USA

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## ABSTRACT

To better understand the mechanisms of plant tolerance to high concentration of arsenic, we characterized two antioxidant enzymes, glutathione reductase (GR) and catalase (CAT), in the fronds of *Pteris vittata*, an arsenic-hyperaccumulating fern, and *Pteris ensiformis*, an arsenic-sensitive fern. The induction, activation and apparent kinetics of GR and CAT in the plants upon arsenic exposure were investigated. Under arsenic exposure (sodium arsenate), CAT activity in *P. vittata* was increased by 1.5-fold, but GR activity was unchanged. Further, GR was not inhibited or activated by the arsenic in assays. No significant differences in  $K_m$  and  $V_{max}$  values of GR or CAT were observed between the two ferns. However, CAT activity in *P. vittata* was activated by 200  $\mu$ M arsenate up to 300% compared to the control. Similar but much smaller increases were observed for *P. ensiformis* and purified bovine liver catalase (133% and 120%, respectively). This research reports, for the first time, the activation of CAT by arsenic in *P. vittata*. The increased CAT activities may allow *P. vittata* to more efficiently mediate arsenic-induced stress by preparing the fern for the impeding production of reactive oxygen species resulting from arsenate reduction to arsenite in the fronds.

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## 1. Introduction

Arsenic contamination of soil and water can result from several anthropogenic activities, such as pesticide use/production, mining, smelting, combustion and sewage/solid waste [6,22]. Currently there are several options that exist for the remediation of arsenic contaminated soils. One option is phytoextraction, which employs hyperaccumulator plants to remove contaminants from soil or water.

Arsenic hyperaccumulator *Pteris vittata* L. (Chinese brake fern) accumulates large amounts of arsenic in its fronds without showing toxicity symptoms [19]. For most plants, arsenic accumulation induces the production of reactive oxygen species (ROS), such as superoxide anions ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals (OH), causing significant injury to plant cells [5,11]. We hypothesize that *P. vittata* has special adaptations to tolerate

**Abbreviations:** GR, Glutathione reductase; CAT, Catalase; AR, Arsenate reductase; ROS, Reactive oxygen species;  $H_2O_2$ , Hydrogen peroxide; GSH, Glutathione; GSSG, Glutathione disulfide; NADPH, Nicotinamide adenine dinucleotide phosphate, reduced form; PEG, Polyethylene glycol.

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oxidative stress, however, the mechanisms of its arsenic tolerance is still not fully understood.

Both enzymatic and non enzymatic antioxidants play significant roles in protecting plant cells from oxidant stress. Among them, catalase (CAT) and glutathione (GSH) are examples of two important antioxidants in plants. Enzymatic antioxidant CAT is one of the most potent catalysts in plants. It catalyzes the conversion of ROS hydrogen peroxide to water and oxygen. Non enzymatic antioxidant GSH is the most abundant non-protein thiol [17]. Its significance lies mostly in its role as a reductant and in its ability to detoxify harmful components within a cell. It is a precursor for phytochelatins, thiol peptides involved in detoxification of heavy metals [23]. Glutathione reductase (GR) is the enzyme that, in conjunction with NADPH, catalyzes the reduction of glutathione disulfide (GSSG) to GSH [3].

The roles of CAT, GSH and GR in plant tolerance to heavy metal stresses have been the subject of several studies [9,21,31]. Srivastava et al. [27] found that *Pteris vittata* and *Pteris ensiformis*, an arsenic-sensitive fern, had similar CAT activities in the fronds (aboveground biomass) in the absence of arsenic. After exposure to 150  $\mu$ M As for 10-d, CAT activity is significantly increased in the fronds of both plants (21–28%). This may imply a role of CAT in arsenic tolerance by both plants. However, under similar condition, GR is not induced by arsenic in either plant. However, Singh et al.

[26] found that *P. vittata* had intrinsically much higher GSH (212%) and GSSG (82%) concentrations in its fronds compared to *P. ensiformis*. This suggests that in *P. vittata* GR activity may not be limiting for maintaining the reduced state of the thiols under arsenic stress.

Several studies revealed that most arsenic in the fronds of *P. vittata* is present as arsenite (AsIII), while most arsenic in its roots is present as arsenate (AsV) [16,32]. Tu et al. [29] showed that most of the arsenic is reduced to arsenite in the fronds of *P. vittata*. The reduction of arsenate to arsenite may lead to the production of cellular ROS, such as H<sub>2</sub>O<sub>2</sub>, because of arsenite binding and inhibiting thiol proteins. Hence, CAT and GR may be important for *P. vittata* to reduce arsenic-induced oxidant stress in the fronds.

Building upon the studies of Srivastava et al. [27] and Singh et al. [26], we characterized two enzymatic antioxidants GR and CAT in the fronds of *P. vittata* and *P. ensiformis* in the presence and absence of arsenic. The objectives of this study were to: 1) determine the impacts of arsenic on GR and CAT activity; 2) compare apparent kinetic constants ( $K_m$  and  $V_{max}$ ) of GR and CAT; and 3) examine the interaction between arsenic (arsenate and arsenite) and enzymes (GR and CAT).

## 2. Materials and methods

### 2.1. Plant and chemical materials

*Pteris vittata* and *P. ensiformis* ferns were used in this study. To ensure uniformity in age and size, all ferns were propagated from spores and cultured in a commercial potting medium as described by Bondada et al. [2]. All chemicals were supplied by Fisher Scientific (Pittsburgh, PA USA) or Sigma (St. Louis, MO USA), unless otherwise stated.

### 2.2. Enzyme induction study

*Pteris vittata* ferns of similar size and age (approximately 90 d after seeding spores) were placed in a hydroponics system and acclimated for 7 d in 0.2-strength Hoagland-Arnon solution [12]. The ferns were kept in a controlled environment with 65% humidity and day and night temperatures of 25 °C and 20 °C, respectively. The ferns were subjected to an 8 h light period with a light intensity of 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Arsenic as sodium arsenate (Na<sub>2</sub>HAsO<sub>4</sub>·7H<sub>2</sub>O) was added to a 0.2-strength Hoagland-Arnon solution with final concentrations of 0 and 10 mg L<sup>-1</sup>.

After 3 d, fern fronds were harvested, flash frozen in liquid nitrogen and stored at -80 °C until analysis. Protein extraction and determination, and GR and CAT enzymatic assays were performed on the 20% PEG (polyethylene glycol) fractions. To rule out inhibitors in the arsenic-treated tissue extracts, GR and CAT activities were also determined in mixed samples using equal amounts of frond tissue from both 0 and 10 mg L<sup>-1</sup> arsenic-treated plants.

### 2.3. Determination of apparent kinetics

The apparent Michaelis–Menten enzyme kinetic parameters,  $V_{max}$  and  $K_m$ , were determined for GR and CAT in *P. vittata* and *P. ensiformis* using the 20% PEG fractions. The assay procedures for GR and CAT were similar to those described above except that substrate concentrations were varied as indicated. All assays were performed in triplicate.

The apparent kinetic parameters for GR were determined for both GSSG and NADPH. The NADPH concentration was fixed at a saturating concentration during GSSG kinetics determination, and the GSSG concentration was fixed at a saturating concentration during NADPH kinetics determination. For CAT, the apparent kinetic parameters of H<sub>2</sub>O<sub>2</sub> were determined.

Data were plotted using Lineweaver–Burk plots (double reciprocal plots). The apparent kinetic parameters were derived from  $x$  ( $-1/K_m$ ) and  $y$  ( $1/V_{max}$ ) intercepts of the plots.

### 2.4. Arsenic effects on enzyme activities

Inhibition and/or activation of GR and CAT activities by arsenic in *P. vittata* and *P. ensiformis* were examined. Various concentrations of arsenic (up to 1000  $\mu\text{M}$ ) were added directly to assays immediately prior to initiation of the enzymatic reaction without altering the pH of the assay. For GR, both arsenate as sodium arsenate and arsenite as sodium arsenite were examined. However, for CAT, only arsenate as sodium arsenate was used to examine inhibition or activation due to arsenite oxidation to arsenate upon exposure to H<sub>2</sub>O<sub>2</sub> [1]. Therefore, the addition of arsenite would give similar results to arsenate. Effects of arsenate on CAT activity of purified bovine liver CAT were also examined for comparison.

### 2.5. Enzyme extraction

Fresh fronds of both ferns with and without arsenic exposure were homogenized in a chilled mortar containing sea sand and extraction buffer as described by Rathinasabapathi et al. [25]. The homogenate was filtered through cheesecloth and centrifuged for 20 min at 20,000g and 4 °C. To concentrate the protein it was precipitated between 5% (wt/v) and 20% (wt/v) PEG. The PEG fraction pellet was dissolved in buffer A (50 mM Tris–HCl pH 8.0, 5 mM DTT and 10% glycerol). All proteins were stored at -80 °C until analysis.

### 2.6. Protein and enzymatic activity determinations

Protein concentrations were estimated in the various fractions using the method of Peterson [24]. Bovine serum albumin (BSA) was used as a standard.

Glutathione reductase (EC 1.6.4.2) activity was assayed by following NADPH oxidation at 340 nm on a UV-spectrophotometer (Beckman Coulter, Fullerton, CA) for 5 min in 1 ml of an assay mixture. The assay contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.15 mM NADPH, 0.5 mM GSSG and 50  $\mu\text{l}$  of enzyme extract. The reaction was initiated by the addition of NADPH [14]. Glutathione reductase activity was calculated using the extinction coefficient of NADPH at 340 nm (6.2 mM<sup>-1</sup> cm<sup>-1</sup>).

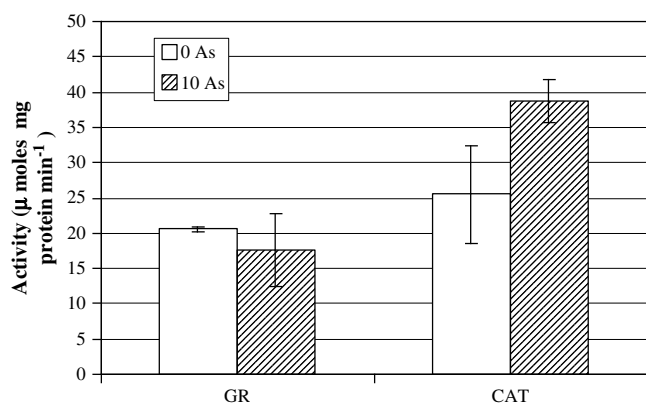
Catalase (EC 1.11.1.6) activity was assayed by following the degradation of H<sub>2</sub>O<sub>2</sub> by absorbance reduction at 240 nm for 3 min with a UV-spectrophotometer [4]. The assay contained 50 mM potassium phosphate buffer (pH 7.0), 88 mM H<sub>2</sub>O<sub>2</sub> and approximately 50  $\mu\text{g}$  protein. The reaction was initiated by the addition of H<sub>2</sub>O<sub>2</sub>. Catalase activity was calculated using the extinction coefficient of H<sub>2</sub>O<sub>2</sub> at 240 nm (40 mM<sup>-1</sup> cm<sup>-1</sup>).

### 2.7. Experimental design and data analysis

The enzyme induction study used a completely randomized design, which consisted of two enzymes (CAT and GR) and two arsenic levels (0 and 10 mg L<sup>-1</sup>). The kinetic studies were done in triplicates. Mean separations were done using *t*-tests at  $p < 0.05$  using SAS statistical software package.

## 3. Results

This study consisted of three sub-experiments using two ferns of *Pteris* genus: *P. vittata* and *P. ensiformis*. In the enzyme induction experiment, *P. vittata* ferns were exposed to 0 and 10 mg L<sup>-1</sup> arsenate for 3-d. In the kinetic determination,  $V_{max}$  and  $K_m$  were



**Fig. 1.** Glutathione reductase (GR) and catalase (CAT) activity in the fronds *P. vittata* after exposing to 0 and 10 mg L<sup>-1</sup> arsenate for 3-d. GR activity was not significantly different among the treatments. CAT activity was significantly ( $P < 0.05$ ) greater in the fronds exposed to 10 mg L<sup>-1</sup> arsenate. Values represent means  $\pm$  std. dev. ( $n = 3$ ).

determined for GR using GSSG and NADPH, and for CAT using H<sub>2</sub>O<sub>2</sub> as substrate in both ferns. In the activation/inhibition experiment, the interactions of GR with arsenate and arsenite, and CAT with arsenate in both ferns were determined.

### 3.1. Arsenic induction of glutathione reductase and catalase

Spectrophotometric assays of GR activity indicated that GR in *P. vittata* was not induced upon arsenic exposure (Fig. 1). In contrast, CAT activity significantly ( $p < 0.05$ ) increased (approximately by 1.5-fold) when the ferns were exposed to arsenic (Fig. 1).

### 3.2. Apparent kinetic of glutathione reductase and catalase

The GR activities exhibited Michaelis–Menten kinetics with respect to the substrate saturation response (Fig. 2). Although the reactions with H<sub>2</sub>O<sub>2</sub> catalyzed by CAT did appear to exhibit Michaelis–Menten kinetics for the substrate concentrations used (Fig. 3), substrate saturation was not reached for either species

(data not shown). Higher H<sub>2</sub>O<sub>2</sub> concentrations could not be used accurately in the spectrophotometric assays.

There were no significant differences between the apparent kinetic constant ( $K_m$ ) of *P. vittata* and *P. ensiformis* enzymes for the substrates (Figs. 2 and 3). The values for  $V_{max}$  of GR were also comparable between the two ferns (Table 1). However, the  $V_{max}$  of CAT activity in *P. ensiformis* was approximately an order of magnitude greater than that in *P. vittata*.

### 3.3. Effect of arsenic on enzyme activities

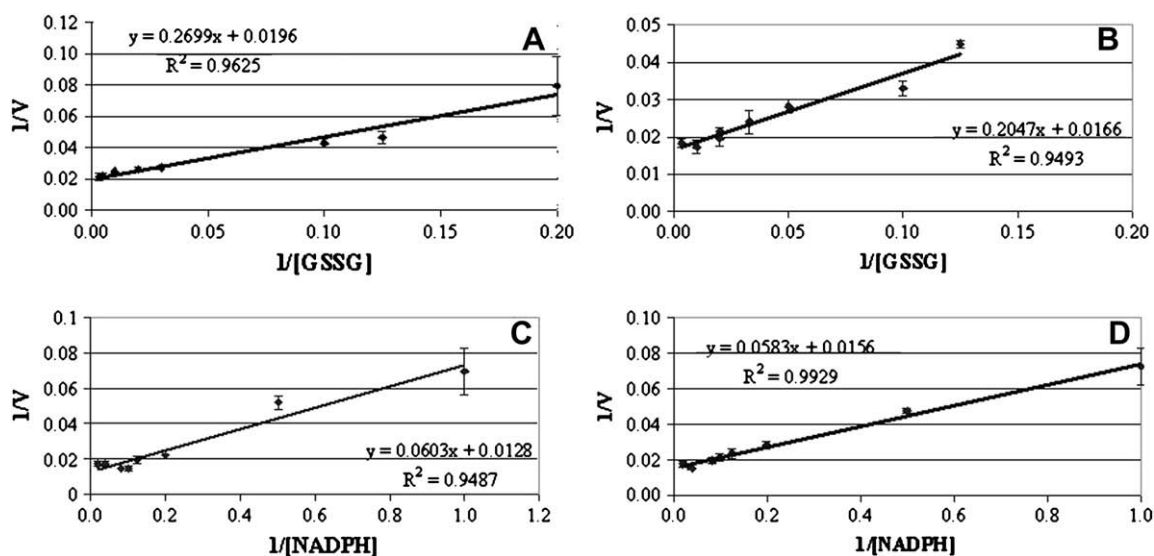
Assays over a range of arsenate and arsenite concentrations, 0–500  $\mu$ M, did not reveal inhibition or an increase of GR activity in the fronds of *P. vittata* or *P. ensiformis* (data not shown). Significant inhibition was not observed in either plant species until  $\geq 1$  mM arsenite was added to the assay. At 1 mM arsenite, GR activity was inhibited approximately by 64% in both ferns. Inhibition by arsenite up to 250  $\mu$ M for both fern species is shown in Fig. 4. Arsenate concentrations up to 3 mM did not inhibit GR activity (data not shown).

Similarly, arsenate (0–1000  $\mu$ M) did not inhibit CAT activity in *P. vittata*, *P. ensiformis* or purified porcine CAT (Fig. 5). On the contrary, the addition of arsenate activated CAT activity in *P. vittata*. The activation pattern in response to different concentrations of arsenate was biphasic. Catalase activity in *P. vittata* increased by 175% at 10  $\mu$ M As (Fig. 5, inset) and returned to the control level at 20  $\mu$ M A. However, it increased again and reached a maximum (300% of the control) at 200  $\mu$ M As before returned to the control level at 500  $\mu$ M As.

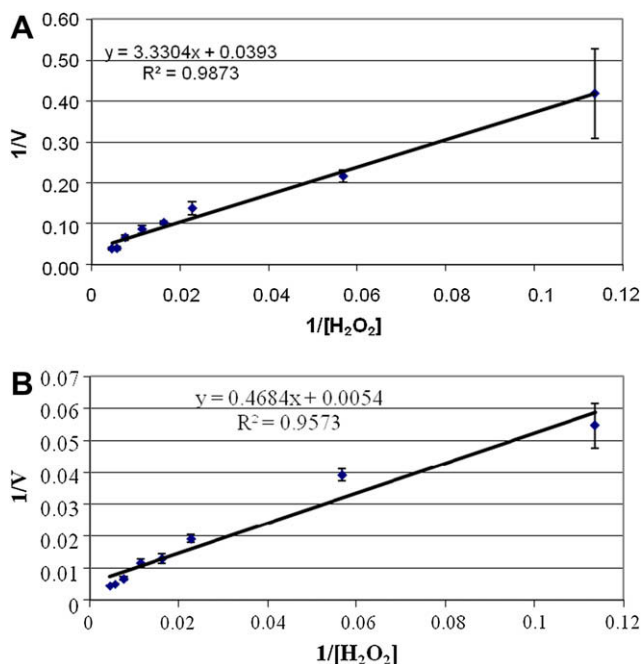
Enzymes from *P. ensiformis* and the bovine liver (positive control) (Fig. 5) showed similar patterns. However, their maximum activity increases were only 133% and 120%, respectively. The maximum CAT activity for both ferns was obtained at 200  $\mu$ M As whereas it was at 100  $\mu$ M As for the bovine liver (Fig. 5).

## 4. Discussion

Antioxidant enzymes play an important role in plant responses to environmental stress including arsenic-induced oxidative stress. Changes in antioxidant enzyme activities upon stress can provide insight into a plant's ability to tolerate stress and mediate its effects.



**Fig. 2.** Lineweaver–Burk plot showing apparent kinetic analysis of substrate GSSG (5–300  $\mu$ M with NADPH = 200  $\mu$ M) for GR activity in *P. vittata* (A) and *P. ensiformis* (B) extracts, and of substrate NADPH (1 and 50  $\mu$ M with GSSG = 100  $\mu$ M) for GR activity in *P. vittata* (C) and *P. ensiformis* (D). Values represent means  $\pm$  std. dev. ( $n = 3$ ).



**Fig. 3.** Lineweaver–Burk plot showing apparent kinetic analysis of substrate  $H_2O_2$  (8.8 and 220 mM) for CAT activity in *P. vittata*. (A) and *P. ensiformis* (B). Values represent means  $\pm$  std. dev. ( $n = 3$ ).

4.1. Glutathione reductase was detected in the fronds of *P. vittata*

Activity of GR was detected in the fronds of *P. vittata* in the presence or absence of arsenic in this study (Fig. 1). Similar results were obtained by Srivastava et al. [27]. However, Duan et al. [7] did not detect GR in the fronds or roots of *P. vittata*. Based on Tu et al. [29], arsenic reduction occurs primarily in the fronds of *P. vittata*. If this is the case, then GR should have a role in the fronds. This is because GR is necessary for the enzyme arsenate reductase (AR) in *P. vittata*, as it reduces GSSG to GSH during glutaredoxin-dependent arsenate reduction [7]. Therefore, AR is hypothesized to play a central role in arsenic hyperaccumulation. The detection of GR activity in the fronds of *P. vittata* is consistent with this hypothesis.

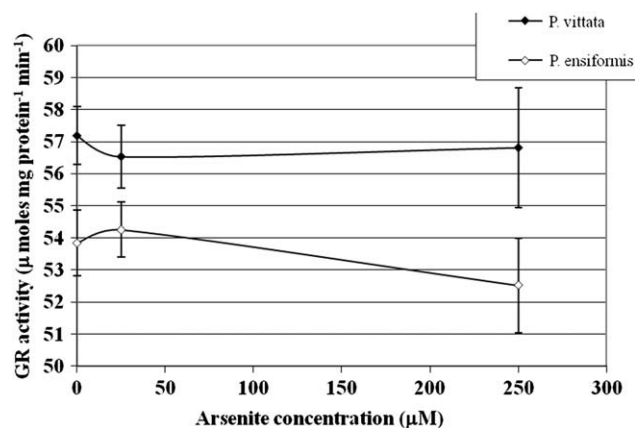
4.2. GR activity was not induced in *P. vittata* upon arsenic exposure

Singh et al. [26] found that the GSH:GSSG ratio did not change significantly in *P. vittata* after exposing to 133–267  $\mu M$  A for up to 15 d. This may imply that GR played a role during the process. However, GR activity was not induced in *P. vittata* upon arsenic exposure (Fig. 1). Therefore, its induction was not necessary for efficient functioning of GR in the fronds of *P. vittata*. These results

**Table 1**

Apparent kinetic parameters for glutathione reductase (GR) and catalase (CAT). Values were derived from Lineweaver–Burk plots for GR substrates (GSSG and NADPH) and CAT substrate ( $H_2O_2$ ) measured in the fronds of *P. vittata* and *P. ensiformis* protein extracts.

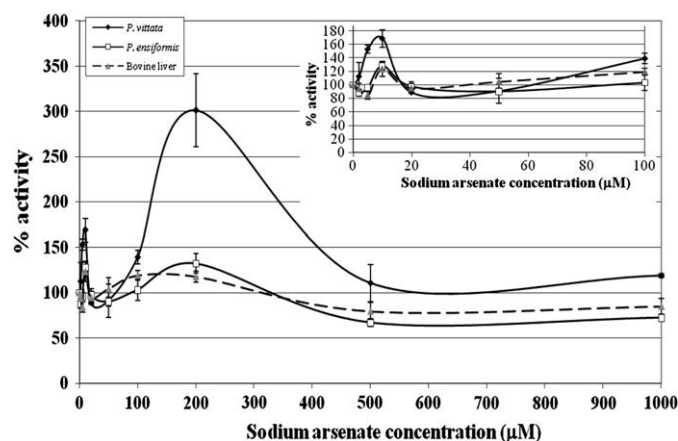
Glutathione reductase	<i>Pteris vittata</i>		<i>Pteris ensiformis</i>	
	$K_m$ ( $\mu M$ )	$V_{max}$ ( $\mu moles\ mg\ protein^{-1}\ min^{-1}$ )	$K_m$ ( $\mu M$ )	$V_{max}$ ( $\mu moles\ mg\ protein^{-1}\ min^{-1}$ )
GSSG	$13.8 \pm 2.2$	$51.0 \pm 1.1$	$12.3 \pm 0.5$	$60.2 \pm 1.8$
NADPH	$4.7 \pm 1.5$	$78.1 \pm 9.6$	$3.7 \pm 1.0$	$64.1 \pm 6.7$
Catalase	(mM)		(mM)	
$H_2O_2$	$84.7 \pm 75.8$	$25.4 \pm 13.2$	$86.7 \pm 31.9$	$185 \pm 40.3$



**Fig. 4.** Effect of arsenite on GR activity in the fronds *P. vittata* and *P. ensiformis*. Values represent means  $\pm$  std. dev. ( $n = 3$ ). Mean comparisons showed that GR activities were not significantly among the three treatments at  $P < 0.05$  for both species.

also indicated that while GSH is an important antioxidant, its recycling from GSSG to GSH by GR was not affected by arsenic. Therefore, *P. vittata*'s ability to maintain the GSH:GSSG ratio is not compromised in the presence of arsenic. The same study indicated that GSH concentrations increased in *P. vittata* upon arsenic exposure [26]. Therefore, it is possible that one or both of the GSH synthesizing enzymes (gamma-glutamyl cysteinyl synthetase and glutathione synthetase) may be induced by arsenic, thereby increasing GSH concentration and maintaining the GSH:GSSG ratio.

It was originally thought that although GR may not be induced in *P. vittata*, it may be more efficient (in terms of  $K_m$ ) compared to a non-arsenic hyperaccumulator, *P. ensiformis*. However,  $K_m$  constants were similar in both ferns (Table 1). These  $K_m$  values were also comparable to those previously reported for GR in other plants, which ranged from 9 to 200  $\mu M$  for GSSG and 1.3–13  $\mu M$  for NADPH [10,30]. The direct plots for NADPH (data not shown) indicated that at higher concentrations the substrate inhibited GR activity. This was more evident in the *P. vittata* enzyme.



**Fig. 5.** Changes in CAT activity in frond protein extracts and bovine liver (positive control) upon addition of arsenate. Percent change is based on the control (no sodium arsenate) assays for each protein. The control activities for *P. vittata*, *P. ensiformis* and bovine liver were 16, 134 and 3271  $\mu mol\ min^{-1}\ mg\ protein^{-1}$ . Values represent means  $\pm$  std. dev. ( $n = 3$ ). The inset shows the activities for lower range of arsenate concentrations (0–100  $\mu M$ ). Mean comparisons showed that arsenic treatments were significantly greater than the control ( $P < 0.001$ ), means of *P. vittata* were significantly greater than that of *P. ensiformis* ( $P < 0.001$ ), and arsenic and plant interaction was highly significant ( $P < 0.001$ ).

#### 4.3. Arsenic did not inhibit or activate GR activity

It is interesting that arsenic did not inhibit or activate GR activity in *P. vittata* or *P. ensiformis* (Fig. 2). Because arsenite can readily bind to compounds with thiol groups, such as GSH, its presence could impact GR activity by altering the GSH:GSSG ratio. Although arsenite did inhibit GR activity in both species, it did so at a concentration (1 mM) that was at least an order of magnitude greater than the substrates, GSSG and NADPH. A lack of inhibition of GR activity by arsenic suggests that GR operates in spite of high arsenic concentrations.

The combined results of the induction, kinetics and inhibition studies suggest that GR is not affected by arsenic in *P. vittata* or *P. ensiformis*. However, further investigation into the GSH synthesizing enzymes, gamma-glutamyl cysteinyl synthetase and glutathione synthetase, may yield interesting results, as GSH is an important component for AR and for detoxification of arsenic in *P. vittata*.

#### 4.4. Arsenic significantly increased CAT activity

Catalase is the enzyme responsible for the degradation of the ROS, H<sub>2</sub>O<sub>2</sub>, to water and oxygen. Unlike GR, CAT was increased by 1.5 times in the fronds of *P. vittata* exposed to arsenic ( $p < 0.05$ ; Fig. 1). Catalase activities were also induced in the fronds *P. vittata* and *P. ensiformis* by Srivastava et al. [27]. *Pteris vittata* has been shown to contain mostly arsenite in its fronds and mostly arsenate in the roots (Tu et al., 2003; [16]). Arsenate is reduced to arsenite mostly in the fronds of *P. vittata* [29]. It is known that the reduction of arsenate to arsenite in plants increases ROS concentration [20]. Therefore, it is possible that the induction of CAT activity may be a result of H<sub>2</sub>O<sub>2</sub> produced, directly or indirectly, from arsenate reduction in the fronds.

Superoxide dismutase (SOD) activity was also induced in *P. vittata* fronds [27]. The SOD enzyme dismutates superoxide anions, producing H<sub>2</sub>O<sub>2</sub> in the process [8]. Therefore, the increase in CAT activity may also be affected by the induction of SOD.

Catalases have been known to be extremely efficient in degrading H<sub>2</sub>O<sub>2</sub>. The determination of the  $K_m$  constants from the CAT kinetics assays did not indicate that CAT enzymes in *P. vittata* were more efficient than *P. ensiformis*. The  $K_m$  values reported in this study were within the range for yeast and bacteria, 64–537 mM [28].

#### 4.5. Significant difference in CAT $V_{max}$ values between two ferns

The 7–8-fold difference in CAT  $V_{max}$  values between the two species is interesting (Table 1). This difference could be due to the presence of an inhibitor in the *P. vittata* extract. It may also be simply due to differences between the two plants. The differences may result from inactivation of smaller-subunit CAT by H<sub>2</sub>O<sub>2</sub> damage [28]. Small-subunit CAT reached their maximum velocity around 200 mM H<sub>2</sub>O<sub>2</sub>, similar to *P. vittata* CAT. However, larger-subunit CAT reached a maximum velocity around 1 M H<sub>2</sub>O<sub>2</sub>. The CAT activity in *P. ensiformis* still appeared to be fairly linear, but with some leveling off at 220 mM H<sub>2</sub>O<sub>2</sub> (data not shown). Determinations of CAT activity with H<sub>2</sub>O<sub>2</sub> concentrations much greater than 220 mM were not possible using the spectrophotometric method, as effervescence by H<sub>2</sub>O<sub>2</sub> does not allow for an observed linear decrease in absorbance. It is unclear why CAT in *P. vittata* appears to be more sensitive to damage by H<sub>2</sub>O<sub>2</sub>. It would be expected that the arsenic hyperaccumulating fern would be less sensitive to such damage. However, because the proteins were not purified, the accuracy of these  $V_{max}$  values is uncertain.

The study by Switala and Loewen [28] also concluded that the traditional Michaelis–Menten kinetics terms,  $K_m$  and  $V_{max}$ , cannot be directly used for CAT. Catalases do not follow Michaelis–Menten kinetics over the H<sub>2</sub>O<sub>2</sub> concentration range because of the two-step CAT reaction. Therefore, the kinetic parameters should be considered to be theoretical. This is especially true for concentrations greater than 200 mM H<sub>2</sub>O<sub>2</sub>. The same study did find a better correlation for lower substrate concentrations, such as those used in this experiment.

#### 4.6. Activation of CAT by arsenate may help *P. vittata* to tolerate arsenic

Certain enzymes, such as mevalonate kinase [18] and nitrate reductase [15] are induced upon arsenic exposure in some plants. Catalase has been shown to be up-regulated by arsenic at the transcriptional level [21]. However, to our knowledge, post-translational regulation of CAT by arsenic has not been reported. This is the first report of CAT activation by arsenate. Sodium arsenate activated CAT activity in *P. vittata* at two concentrations: 10 and 200  $\mu$ M (Fig. 5). The percent activation at 200  $\mu$ M As was approximately 1.8 times greater than that at 10  $\mu$ M As. The activation of CAT activity with maxima at two concentrations of arsenic is consistent with the presence of two different CAT isozymes with differing arsenic activation properties. Different CAT isozymes have been shown to respond differently to the same conditions or stresses in other plants [13]. Similar activation patterns, although less pronounced, were observed in *P. ensiformis* and the purified bovine liver CAT suggesting that activation of CAT by arsenate is highly conserved.

It is possible that the presence of arsenate activates some CAT isozymes in preparation for the pending arsenate reduction and the subsequent production of H<sub>2</sub>O<sub>2</sub>. Therefore, these results suggest that activation of CAT by arsenate may constitute an important role in the ability of *P. vittata* to tolerate high levels of arsenic.

The results suggested that CAT may be important in arsenic-hyperaccumulation in *P. vittata*. Catalase was not only induced in the fronds of *P. vittata*, but it was also activated, up to 300% upon arsenic exposure. Although a similar activation pattern was observed in the non-hyperaccumulator, *P. ensiformis*, the magnitude was much smaller. The activation of CAT at two different concentrations of arsenate may be the result of different CAT isozymes present in the fern. It was hypothesized that the CAT induction by arsenate had an adaptive role in preparing *P. vittata* for ROS species resulting from arsenate reduction in the fronds.

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#### References

- [1] H.V. Aposhian, R.A. Zakharyan, M.D. Avram, M.J. Kopplin, M.L. Wollenberg, Oxidation and detoxification of trivalent arsenic species, *Toxicol. Appl. Pharmacol.* 193 (2003) 1–8.
- [2] B.R. Bondada, S. Tu, L.Q. Ma, Absorption of foliar-applied arsenic by the arsenic hyperaccumulating fern (*Pteris vittata* L.), *Sci. Total Environ.* 332 (2004) 61–70.
- [3] I. Carlberg, B. Mannervik, Glutathione reductase, in: A. Meister (Ed.), *Glutamate, Glutamine, Glutathione, and Related Compounds*, Academic Press, Inc., Orlando, FL, 1985, pp. 484–490.
- [4] B. Chance, A.C. Maehly, Assay of catalases and peroxidases, *Methods. Enzymol.* 2 (1955) 764–817.
- [5] P.L. Conklin, Recent advances in the role and biosynthesis of ascorbic acid in plants, *Plant Cell Environ.* 24 (2001) 383–394.
- [6] A. Davis, D. Sherwin, R. Ditmars, K.A. Hoenke, An analysis of soil arsenic records of decision, *Environ. Sci. Technol.* 35 (2001) 2401–2406.

- [7] G.L. Duan, Y.G. Zhu, Y.P. Tong, C. Cai, R. Kneer, Characterization of arsenate reductase in the extract of roots and fronds of Chinese brake fern, an arsenic hyperaccumulator, *Plant Physiol.* 138 (2005) 461–469.
- [8] I. Fridovich, Superoxide radical and superoxide dismutase, *Annu. Rev. Biochem.* 64 (1995) 97–112.
- [9] M. Gupta, A. Cuypers, J. Vangronsveld, H. Clijsters, Copper affects the enzymes of the ascorbate–glutathione cycle and its related metabolites in the roots of *Phaseolus vulgaris*, *Physiol. Plant* 106 (1999) 262–267.
- [10] N. Hakam, J.P. Simon, Molecular forms and thermal and kinetic properties of purified glutathione reductase from two populations of barnyard grass (*Echinochloa crus-galli* (L.) Beauv.: Poaceae) from contrasting climatic regions in North America, *Can. J. Bot.* 78 (2005) 969–980.
- [11] J. Hartley-Whitaker, G. Ainsworth, A.A. Meharg, Copper and arsenate induced oxidative stress in *Holcus lantus* L. clones with differential sensitivity, *Plant Cell Environ.* 24 (2001) 713–722.
- [12] D.R. Hoagland, D.I. Arnon, The Water Culture Method for Growing Plants Without Soil, California Agril. Exp. Sta, 1938, Circular No. 3. 346–347.
- [13] E. Horvath, T. Janda, G. Szalai, E. Paldi, In vitro salicylic acid inhibition of catalase activity in maize: differences between the isozymes and a possible role in the induction of chilling tolerance, *Plant Sci.* 163 (2002) 1129–1135.
- [14] M. Jiang, J. Zhang, Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves, *J. Exp. Bot.* 53 (2002) 2401–2410.
- [15] C.J. Kay, M.J. Barber, EPR and kinetic analysis of the interaction of halides and phosphate with nitrate reductase, *Biochemistry* 28 (1989) 5750–5758.
- [16] G.M. Kertulis, L.Q. Ma, G.E. MacDonald, R. Chen, J. Winefordner, Y. Cai, Arsenic speciation and transport in *Pteris vittata* L. and the effects on phosphate in the xylem sap, *Environ. Exp. Bot.* 54 (2005) 239–247.
- [17] R. Kneer, M.H. Zenk, Phytochelatins protect plant enzymes from heavy metal poisoning, *Phytochemistry* 31 (1992) 2663–2667.
- [18] C.S. Lee, W.J. O'Sullivan, An improved purification procedure, an alternative assay and activation of mevalonate kinase by ATP, *Biochim. Biophys. Acta* 747 (1983) 215–224.
- [19] L.Q. Ma, K.M. Komar, C. Tu, W. Zhang, Y. Cai, A fern that hyperaccumulates arsenic, *Nature* 409 (2001) 579.
- [20] A.A. Meharg, J. Hartley-Whitaker, Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species, *New Phytol.* 154 (2002) 29–43.
- [21] P.V. Mylona, A.N. Polidoros, J.G. Scandalios, Modulation of antioxidant responses by arsenic in maize, *Free Radic. Biol. Med.* 25 (1998) 576–585.
- [22] R.S. Oremland, J.F. Stolz, The ecology of arsenic, *Science* 300 (2003) 939–944.
- [23] B. Pawlik-Skwronska, Phytochelatin production in freshwater algae *Stigeoclonium* in response to heavy metals contained in mining water; effects of some environmental factors, *Aquat. Toxicol.* 52 (2001) 241–249.
- [24] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, *Anal. Biochem.* 83 (1977) 346–356.
- [25] B. Rathinasabapathi, W.M. Fouad, C.A. Sigua, Alanine betine synthesis in the Plumbaginaceae. Purification and characterization of a trifunctional, S-adenosyl-L-methionine-dependent N-methyltransferase from *Limonium latifolium* leaves, *Plant Physiol.* 126 (2001) 1241–1249.
- [26] N. Singh, L.Q. Ma, M. Srivastava, B. Rathinasabapathi, Metabolic adaptations to arsenic-induced oxidative stress in *Pteris vittata* L. and *Pteris ensiformis* L. *Plant Sci.* 170 (2006) 274–282.
- [27] M. Srivastava, L.Q. Ma, N. Singh, Antioxidant responses of hyperaccumulator and sensitive fern species to arsenic, *J. Exp. Bot.* 56 (2005) 1335–1342.
- [28] J. Switala, P.C. Loewen, Diversity of properties among catalases, *Arch. Biochem. Biophys.* 401 (2002) 145–154.
- [29] S. Tu, L.Q. Ma, G.E. MacDonald, B. Bhaskar, Arsenic absorption, speciation and thiol formation in excised parts of *Pteris vittata* in the presence of phosphorus, *Environ. Exp. Bot.* 51 (2004) 121–131.
- [30] B. Turner, C.J. Pollock, The effects of temperature and pH on the apparent Michaelis constant of glutathione reductase from maize (*Zea mays* L.), *Plant Cell Environ.* 16 (1993) 289–295.
- [31] A.P. Vitoria, P.J. Lea, R.A. Azevedo, Antioxidant enzymes responses to cadmium in radish tissues, *Phytochemistry* 57 (2001) 701–710.
- [32] W. Zhang, Y. Cai, C. Tu, L.Q. Ma, Arsenic speciation and distribution in an arsenic hyperaccumulating plant, *Sci. Total Environ.* 300 (2002) 167–177.