Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 20(2008) 1463–1468
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Arsenic speciation for the phytoremediation by the Chinese brake fern, *Pteris vittata*

R. Shoji*, R. Yajima, Y. Yano

Department of Chemical Science and Engineering, Tokyo National College of Technology, 1220-2 Kunugida, Hachioji, Tokyo 193-0997, Japan. E-mail: shoji@tokyo-ct.ac.jp

Received 20 December 2007; revised 1 February 2008; accepted 21 February 2008

Abstract

Arsenic (As) speciation for the phytoremediation by the Chinese brake fern was studied. In particular, the mechanism of how plants induce compounds containing thiol (SH) and proteins by As exposure in terms of the relationship between As and phosphate uptaken into plant cells was examined. *Pteris vittata* callus could efficiently reduce As(V) to As(III) by the rapid introduction of reductase and synthesize thiols leading to phytochelatins production. Furthermore, *Pteris vittata* could control phosphate concentration in the cells corresponding to the concentration of arsenite and arsenate. To our best knowledge, this is the first report to show the mechanisms of such high As tolerance of *Pteris vittata* using their callus in terms of *in vitro* approach for the analysis of As speciation and metabolism route.

Key words: arsenic hyper accumulator; phosphorus; thiol; phytochelatin

Introduction

In recent years, Arsenic (As) pollution in several areas of the world has caused serious problems. Phytoremediation technique has been noticed as a promising method to reduce As concentration in contaminated areas such as part of India and Bangladesh. Also, high metal tolerance plants, which can hyper-accumulate As, have been examined to be useful for phytoremediation. The Chinese brake fern called *Pteris vittata* can accumulate As in its organ up to 200 times higher than the soil level in terms of As concentration. Considerable research has been focused on assessing As tolerance in this plant, and evolution of As has been well documented in numerous publications. In the greenhouse experiment using artificial As contaminated soil, the As concentration in leaves of the fern reached 22,630 mg/kg (Ma *et al*., 2001). The fern hyperaccumulates some forms of As (ex. arsenate, arsenite, organic As). Most of As accumulated in the fern is arsenite in the stalks and leaves, and some of the As accumulated mainly in roots arsenate. The order of As amount uptaken is leaf > frond > root.

Arsenic, a ubiquitous poison on the Earth, reacts strongly with thiol (SH) in organisms, therefore, has high toxicity. In terms of detoxifying matter of As, several plants have phytochelatins (PCs) consisting of glutathione (GSH), a tripeptide containing cystein having SH (thiols), and its structure is [γ-Glu-Cys-]$_n$-Gly (n: 2–11). The PCs are biosynthesized by peptide transformation of γ-glutamyl-cysteinyl dipeptide based on GSH using PC syntases. It is well-known that PCs can detoxify As (Ha *et al*., 1999), and the reaction mechanism can be described that PCs-As bond is unstable against weak alkaline and stable against weak acid (Schmöger *et al*., 2000). It is also well-known that a compound containing SH was induced by As exposure to the fern, even its SH/As ratio is very low (Hartley-Whitaker *et al*., 2001). According to Zhang *et al*. (2004), most of As should be accumulated in the vacuole of the fern cells. In various kinds of plants, it is known that arsenate is uptaken into inside of the cells by phosphate transporters. It is therefore demonstrated that such plants show certain amount of As tolerance derived by controlling phosphate uptake, which simultaneously cause arsenate uptake. According to current studies, it is suggested that arsenite can also be uptaken through glycerol channel. Besides, the uptake rate of As is affected by coexisting phosphorus concentration. According to Wang *et al*. (2002), the uptake rate of arsenate was 10 times faster than that of arsenite when exposed to arsenite or arsenate without phosphorus. The uptake rate of arsenate decreased with increase in the concentration of phosphate. In contrast, it is suggested that As uptake increased by phosphate addition (Cao and Ma, 2003). Wang *et al*. (2002) showed that the amount of arsenite uptaken was more than that of arsenate for over 2 weeks of exposure time, though the uptake rate of arsenite was less than that of arsenate. Thus, it has never been elucidated on how the interaction between phosphorus and As affects the kinetics

* Corresponding author. E-mail: shoji@tokyo-ct.ac.jp.

or equilibrium of As uptaken into plant cells.

Since the relationship between phosphorus and As is still unclear, it is difficult to distinguish the intermolecular effects on translocation or influx of arsenite and arsenate using whole plant body. There are, however, very few reports on *in vitro* examination of *Pteris vittata* to elucidate the mechanism of high As tolerance. *In vitro* cell experiment provides various distinct advantages such as homogeneity of test sample, independency of location in the plant in a relatively short period. The objective of this study is to examine *in vitro* the reason why *Pteris vittata* has such a high tolerance and accumulating capacity against As. In particular, the mechanism of how the plant induces compounds containing SH and proteins by As exposure in terms of the relationship between As and phosphate uptaken into plant cells was examined.

1 Materials and methods

1.1 Fern sample

Fern spore sample used in this study was supplied from the Laboratory of Soil Science and Plant Nutrition, Mie University, Japan. The fern spores were inoculated and germinated on Murashige-Skoog medium (MS media, Murashige and Skoog, 1962) supplemented with 1% Agar. The final concentration of phosphate acid in the medium was 116 g/L. The fern samples were cultured for 1, 2, 3 months at 24 ± 2°C under continuous lighting on the MS medium containing 0, 23, 45, 68, 91 mg/kg As(III) or As(V), respectively. Ferns were cryopreserved with liquid nitrogen and kept in a freezer at –27°C until analysis, after measuring the weight of the fern harvested at each culture time. Residual media was preserved in a fridge at 4°C until use.

We also measured the cytoplasm pH in fern cells using a micro electrode (< 1.0 μm) pH meter made by the authors. The pH measured by the micro electrode pH meter shows good correlation (R^2 = 0.984) with the pH measured using a conventional pH electrode (MM60R, TOA DKK, Japan) in the pH range of 6.0–9.0.

1.2 As extraction and speciation analysis

As accumulated in fern body was extracted by centrifugation at 25,200 ×*g*, 4°C for 15 min after shaking the homogenization at 150 r/min for 1 h. To homogenize the fern sample, 1 ml of 6 mol/L HCl was added to 0.1 g fern sample and mixed using a mortar and pestle. Then, the supernatant prepared by centrifugation was collected to analyze As. The As was extracted again from the residual fern sample by repeating the same procedure as described above. Then, the primary and secondary supernatants were mixed and filtered through a 0.2-μm membrane filter. The As speciation analysis was performed using Hydride Generation-Atomic Absorption Spectrometry (HG-AAS).

1.3 HG-AAS measurement

An atomic absorption spectrophotometer SAS-7500 (Seiko Instrument, Japan) was used in combination with a hydride generator THG-1200 (Seiko Instrument, Japan) with Argon gas as carrier. The sample flow rate was set at 8 ml/min, and the flow rate of the solution of 10% $NaBH_4$ prepared just prior to the operation of THG-1200 and of 2.4 mol/L HCl was set as 10 ml/min. The flow rates of carrier gas and purging gas were 0.5 and 0.4 ml/min, respectively.

1.4 Total thiol assay

The total HS in plant sample was assayed according to de Vos *et al*. (2004). Nonprotein HS were extracted by homogenation of 20–40 mg fresh weight with 2 ml 5% (*W/V*) sulfosalicylic acid, 6.3 mmol/L diethylenetriaminepentaacetic acid (DTPA, pH < 1) in ice bath. After centrifugation at 25,200 ×*g* for 10 min under 4°C, the supernatants were assayed immediately. The level of total acid-soluble thiols was determined using Ellman's reagent (Ellman, 1959). The supernatant was mixed with 630 μl of 0.5 mol/L K_2HPO_4 to adjust the final pH to 7.5 and the absorbance, A_{412}, was measured at 412 nm using a spectrophotometer (UV-1200, Shimadzu, Japan). After addition of 25 μl of 5,5′-dithiobis (2-nitrobenzoic acid, DTNB) solution consisting of a mixture of 6.0 mmol/L DTNB in 0.143 mol/L K_2HPO_4 and 6.3 mmol/L DTPA, the A_{412} was measured again after 2 min (ε_{TNB} = 13,600 L/(mol·cm)). The increase in absorbance was corrected for the absorbance of DTNB. The total SH concentration was calculated from that increase of A_{412} using the molecular extinction coefficient, 13,600 L/(mol·cm) .

Phosphorus in residual medium was measured using the molybdenum blue method. Sample was filtered by 0.45 μm of membrane filter. Organic matter in the medium solution was decomposed beforehand using a mixture of nitric and sulfatefic acids.

1.5 Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE)

The fern sample cultured for 20 d in medium containing 0 and 91 mg/L of As(III) or As(V) was used for SDS-PAGE. The harvested fern samples were homogenized with 1 ml/0.1 g fern sample of TNE buffer (10 mmol/L Tris-HCl, 100 mmol/L NaCl, 1 mmol/L EDTA) or 0.1 mol/L phosphate buffer (0.1 mol/L K_2PO_4, 100 mmol/L KCl, 5 mmol/L EDTA) on ice bath. The homogenate was centrifuged at 25,200 ×*g*, 15 min, 4°C, and the supernatant was centrifuged in the same condition again. Then, 50 μl of SDS-PAGE samples were mixed with 50 μl of two times concentrated sample buffer (100 mmol/L Tris-HCl, 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, and proper volume of 1% Bromophenol Blue (BPB)) and then boiled for 3 min at 100°C. For coloring the sample, Coomassie Brilliant Blue (CBB) (SeePico CBB Stain Kit, Funakoshi Co., Ltd., Japan) was used.

2 Results

2.1 Growth of fern

The growths, which are described as weight of fern increase along with culture time on MS medium containing

0, 23, 45, 68, 91 mg/L As(III) or As(V) are shown in Fig.1. On the contrary to our expectation, the growth was independent of the concentration of both arsenite and arsenate in medium. No phytotoxic effect on the fern caused by As(III) and As(V) was observed in the concentration range tested in this study as noted in various publications.

2.2 Determination of As by HG-AAS in fern plant

The kinetics of accumulated As in fern samples cultured on MS medium containing As are shown in Fig.2. The As concentration in the fern increased with increase in As concentration, there was no significant difference of As concentration in the fern among fern samples in each concentration of arsenite and arsenate according to the T-test results. The total amount of accumulated As, however, increased with increase in culture time without exceptions. When the As medium concentration was about 70 mg/L, the 1-month As(III) sample had a higher As content in fern than 2- and 3-month. In terms of speciation of As accumulated in the fern, the concentrations of As(III) and As(V) individually analyzed in fern cultured for each terms are shown in Fig.3. It is suggested that As(III) is dominant in the total accumulated As in the fern even though the ferns were exposed to only arsenate as As in the culture medium.

2.3 Total SH assay in the fern cells

Figure 4 shows the kinetics of the total SH concentration in fern cultured in different As concentration. It is clearly that the total SH increased with increase in the concentrations of both As regardless of which As was exposed to the fern.

Figure 5 shows the relationship between the total SH in fern and As concentration in the same fern sample throughout the culture term. By single regressional analysis, the relationship between them can be described as linear. The correlation coefficients were 0.651, 0.668, and 0.673 for 1, 2, 3 months, respectively.

Generally, it is considered that As is mainly detoxified by chelating with compounds having SH (Reaction (1)).

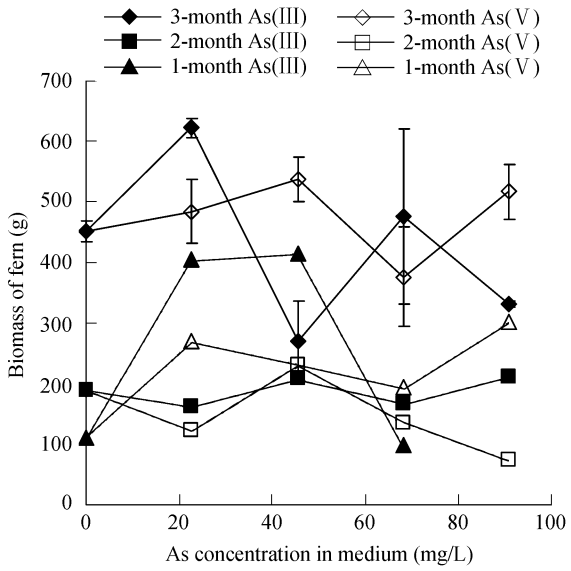


Fig. 1 Effects of different arsenite or arsenate levels and culture terms on the biomass of Chinese brake. Bars represent SD of two replicates.

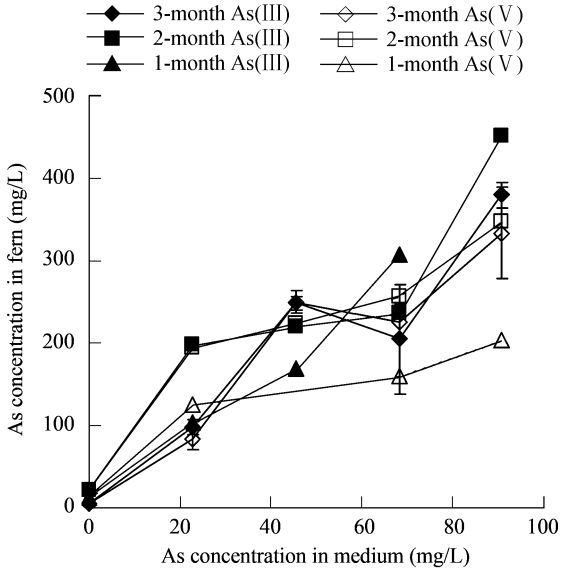


Fig. 2 Effects of different arsenite or arsenate levels and culture terms on the total As concentration of Chinese brake grown on MS medium. Bars represent SD of two replicates.

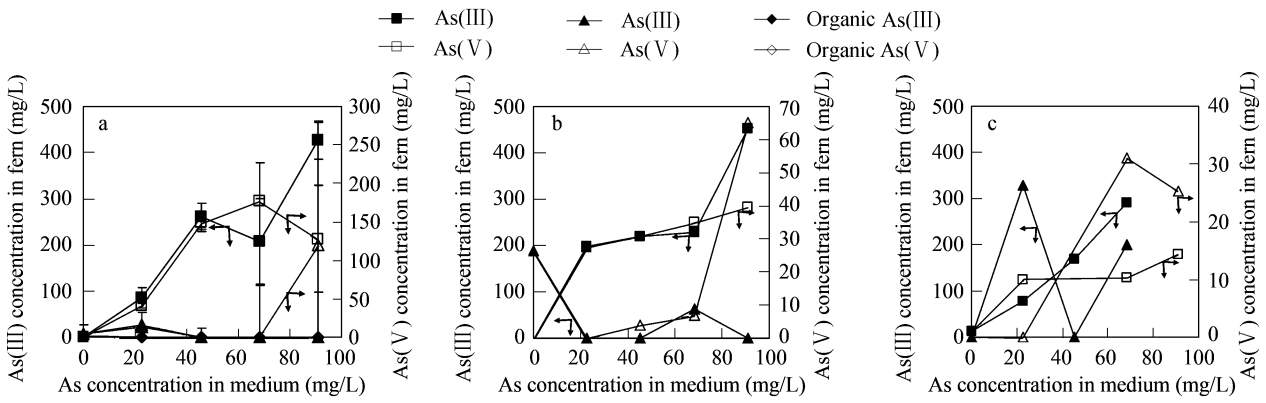


Fig. 3 Effects of different arsenite or arsenate levels on the different form As concentration of Chinese brake cultured for 3 (a), 2 (b), and 1 (c) months on MS medium. Bars represent SD of two replicates.

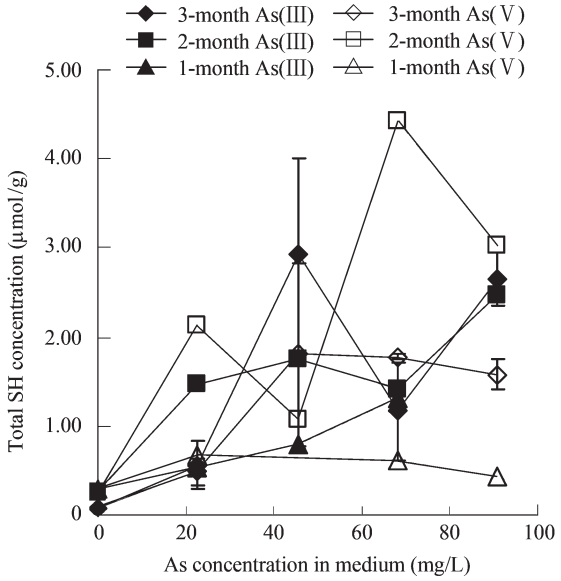
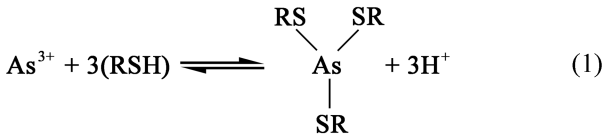


Fig. 4 Effects of different arsenite or arsenate levels and culture terms on the total SH concentration of Chinese brake grown on MS medium. Bars represent SD of two replicates.



The molecular ratio of SH to As should ideally be 3 to 1 to detoxify As completely. It was reported that the SH/As ratio of brake ferns having strong As tolerance was considerably less than 3 (Cai *et al*., 2004). According to the results, the SH/As ratio in the fern was also considerably less than 3, which agreed well with Cai's results.

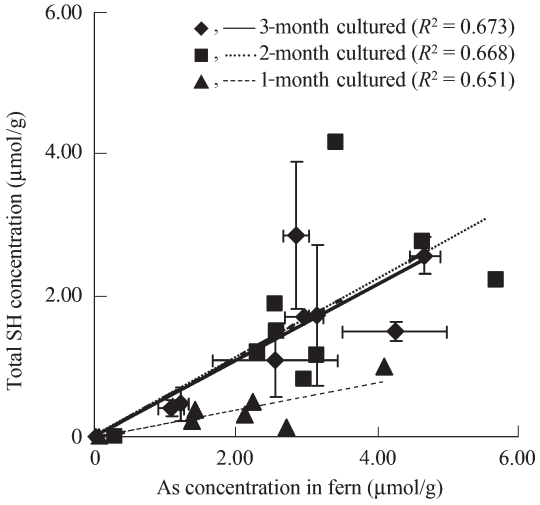


Fig. 5 Effects of different As levels of Chinese brake and culture terms on the total SH concentration of the fern grown on MS medium.

2.4 Phosphorus analysis

The amount of phosphorus absorbed in the fern samples can be calculated by subtracting the measured residual phosphorus in medium from the amount of initially added phosphorus. The relationships between the amount of absorbed phosphorus in fern and the As concentrations in medium or in fern are shown in Fig.6. The amount of absorbed phosphorus decreased with increase in the As concentration in medium or in fern exposed to As(III) or As(V).

2.5 SDS-PAGE

As a result of SDS-PAGE (acryl amide concentration is 10%), the band of protein about 57 kDa were obtained. The intensity of the band of fern sample cultured in medium containing arsenate was indicated more clearly than that in

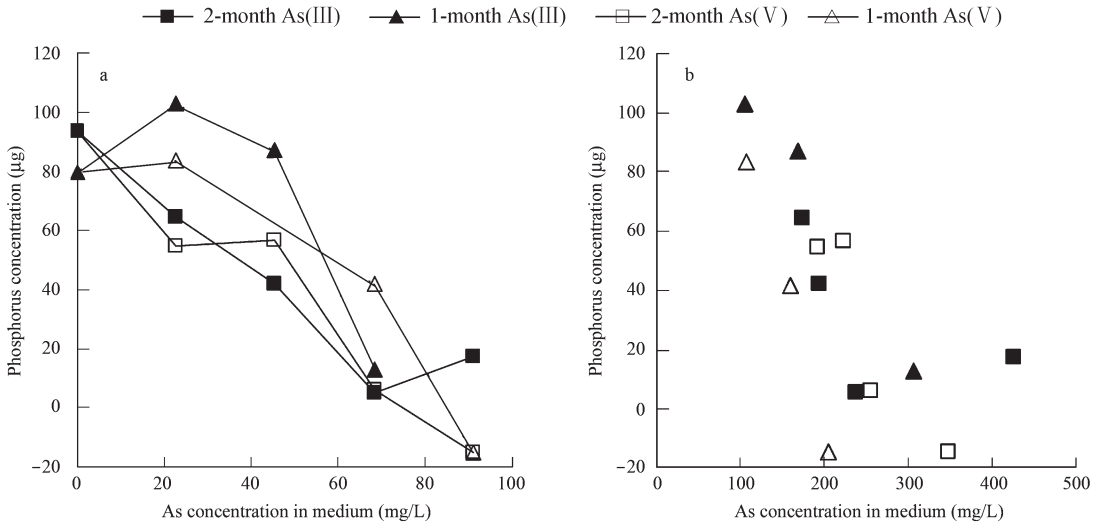


Fig. 6 Effects of different arsenite or arsenate levels of cultured medium (a) and of Chinese brake (b) on the phosphorus concentration of the fern grown in MS medium.

medium containing arsenite. It is obvious that the protein of about 57 kDa was derived by culturing fern on arsenate added medium. On the other hand, the 57 kDa protein was not derived in fern cultured in arsenite containing medium. Therefore, it is clear that the 57 kDa protein does not contribute to the detoxification of arsenite, however, it is related to the reduction of arsenate to arsenite in fern cells.

3 Discussion

The experiments in this study have examined the mechanism of high tolerance of *Pteris vittata* against As *in vitro* in terms of As-induced phytochelatins and SH group. It has been quantitatively examined and compared to the exposed concentration of arsenate and arsenite. The fern sample cultured for one month grew more and absorbed phosphorus more than other fern samples because primary seeding amount of that sample is bigger than others.

The SH/As molecular ratio to detoxify As by PCs must be 3, theoretically. The detoxification of As in the brake fern should be promoted via other molecules except PCs because the ratio of SH/As is considerably less than 3. The result of SDS-PAGE suggested that some proteins except PCs in the fern are not related to the detoxification of As. It is elucidated by our results that specific proteins (57 kDa) were induced by As(V) addition to culture medium while no specific protein was induced by As(III) addition. It is reasonable that this protein plays a role in the reduction of As(V) to As(III) in fern cells, taking consideration of the results of As speciation in the cell. It has already been reported that arsenate reductase was found in the extract of roots and fronds of Chinese Brake Fern, *Pteris vittata* (Duan *et al*., 2005). Although there is no mention of the molecular weight of arsenate reductase, the activity of the enzyme should increase with increase in GSH concentration as indicated by the findings.

In terms of another reason for why *Pteris vittata* has such high tolerance against As, cellular pH in the brake fern (pH = 7.96 under no As exposure) is higher than in several other As sensitive plants (pH = 7.2, generally), and arsenic speciation and stability are focused in the high pH range. Schmöger *et al*. (2000) reported that the SH-As chemical bond between PC_2 (Phytochelatin $n = 2$) and As is stable in relatively low pH range (pH < 6), and becomes unstable in the high pH range (pH > 8). Arsenic phytotoxicity is the inhibition of the activity of proteins in organisms by binding hardly with SH group in proteins, then it can be supposed that the chemical bond between As and SH of concerned proteins in organisms will also become unstable in high pH, because both SH group in PCs and proteins are of cystein origin. The high cellular pH of *Pteris vittata* led to obtain relatively high As tolerance.

Zhang *et al*. (2004) reported that the fern accumulate As mainly in the vacuole. Therefore, it is supposed that As flows into vacuole as arsenite ion through ion channels on the tonoplast and that the arsenite ion ($H_2AsO_3^-$ at pH 7.96) changes to non-dissociative state (H_3AsO_3) accumulated in vacuole because the inside of vacuole is more acidic, therefore, As cannot be released from vacuole to cytoplasm through the ion channel again. Besides, it can also be supposed that if the cellular pH is higher, arsenite can easily dissociate and accumulate in vacuole. In other words, the residence time of As in cytoplasm becomes shorter in higher cellular pH so that the phytotoxicity of As is weakened. Thus, higher cytoplasm pH should be one reason for the As tolerance after all.

However, even the As-SH bond is unstable for high cytoplasm pH, As can combine with SH when the concentrations of As and SH are high enough to proceed the binding reaction as described in Reaction (1). At the same time, it was observed that the SH concentration in the fern samples increased with increase in As concentration in the fern samples and with increase in culture terms. From our results of the kinetics examination in terms of SH and As in the fern samples, it is supported that the higher As tolerance is owing to the higher cellular pH. Further study focused on vacuole pH can reveal the reason why *Pteris vittata* has such high As tolerance.

When arsenite ion is accumulated in the fern vacuole owing to being in non-dissociative state after flowing into the vacuole following that the arsenite flowed into the fern's cytoplasm have dissociated to arsenite ion, the cytoplasm pH will decrease owing to be exhausted H^+ by arsenite, flowed into cytoplasm, dissociating. The vacuole pH will rise due to be consumed H^+ by arsenite ion, taken in vacuole, becoming to non-dissociative state. It can be expected that H^+-ATPase on tonoplast will activate to buffer the pH change and to keep cytoplasm pH higher. So far, there is no literature to report that As inhibits ATPase in the low concentration range. In fact, it has been reported that ATPase can discharge several kinds of toxic heavy metals to outside of the cells. ATPase was synthesized more in some bacteria as toxic metals were exposed to the bacteria (Silver *et al*., 1996). Several kinds of ATPase in this study may have different structures with H^+-ATPase, though this study also suggested that ATPase was not inactivated by low concentration As exposure. H^+-ATPase consumes one ATP molecule for the transportation of H^+. ATP was decomposited to ADP and phosphoric acid (Pi), so that Pi should be emitted simultaneously when H^+-ATPase transports H^+. Therefore, it is supposed that Pi concentration in cytoplasm will increase with taking arsenite into vacuole. It can be said that Pi in cytoplasm is exhausted to outside of the cells to adjust the Pi concentration. This is why the amount of phosphorus uptake from medium into fern cells showed minus value as shown in Fig.6. Quantitative kinetics analysis of the concentration of As and phosphate inside and outside of the fern cells showed that the absorption amount of phosphate decreased with increase in the concentration of As inside and outside of the fern cells. Of the recent publications, the effect of arsenic species and phosphorus on arsenic absorption was examined *in vivo* using *Lupinus albus* L. fern whole body by Reina *et al*. (2005). They showed that the phosphorus deficiency increased the As concentrations in plant tissues, causing an increase in PC accumulation. Although there should be some differences between the two species, our

findings in the fern cellular analysis agreed well with their results. In addition, it is believed that phosphorus plays an important role in As accumulation and detoxification since phosphate and arsenate are analogous (Tu and Ma, 2003).

As discussed above, a certain amount of phosphate must be excreted to outer-cell system to adjust the phosphate concentration in the fern cytoplasm. On the other hand, we assume that phosphate was not emitted from the fern root to soil in the case of *in vivo* test using whole fern because of the essentialness of phosphate to plant healthy growth. Tu and Ma (2003) showed that As(III) was uptaken more than As(V), while we showed that the uptaken amount of As(V) was almost the same as that of As(III). This contradiction can be accounted by the difference between the *in vivo* test by Tu and Ma (2003) and the *in vitro* test performed in this study. From the results of phosphate absorption, it was found that the brake fern callus did not absorb phosphate, and the effect of As(III) on the decrease of absorbed phosphate was almost the same as that of As(V) in the case of cellular level. However, as a whole plant body of *Pteris vittata*, the root can absorb phosphate because the concentration of As(V) accumulated in root tissue is significantly higher than that of As(III) as shown by Poynton *et al*. (2004).

Also, it can be explained that As(V) was absorbed more than As(III) at the short term As exposure, while As(III) was absorbed more than As(V) at long term As exposure as shown previously (Wang *et al*., 2002). Arsenate and/or phosphorus absorption should be suppressed along with the increase in As exposure time because of the increase of phosphorus and As concentration in the fern.

4 Conclusions

In vitro examination performed in this study using fern callus revealed the reason why *Pteris vittata* accumulates As to extremely high concentration as follows:

(1) *Pteris vittata* callus could efficiently reduce As(V) into As(III) by the rapid introduction of reductase and synthesize thiols leading to phytochelatins production.

(2) *Pteris vittata* could control the phosphate concentration in the cells corresponding to the concentration of arsenite and arsenate.

To our best knowledge, this is the first report to indicate the mechanisms of such high As tolerance of *Pteris vittata* using their callus in terms of *in vitro* approach for analysis of As speciation and metabolism route. Further study on cellular pH of the fern cells must be done to reveal the mechanisms of As accumulation in vacuoles.

References

Cai Y, Su J, Ma L Q, 2004. Low molecular weight thiols in arsenic hyperaccmulator *Pteris vittata* upon exposure to arsenic and other trace elements. *Environmental Pollution*, 129: 69–78.

Cao X, Ma L Q, 2003. Effects of compost and phosphate on plant arsenic accumulation from soil near pressure-treated wood. *Environmental Pollution*, 132: 435–442.

de Vos F, Willemse P, de Vries E, Gietema J A, 2004. Endothelial cell effects of cytotoxic: balance between desired and unwanted effects. *Cancer Treatment Reviews*, 30: 495–513.

Duan G L, Zhu Y, Tong Y, Cai C, Kneer R, 2005. Characterization of arsenate ruductase in the extract of roots and fronds of Chinese Brake Fern, an arsenic hyperaccumulator. *Plant Physiol*, 138: 461–469.

Ellman G L, 1959 Tissue sulphydryl groups. *Arch Biomed Biophys*, 82: 70–77.

Ha S B, Smith A P, Howden R, Dietrich W M, Bugg S, O'Connell M J, Goldsbrough P B, Cobbett C S, 1999. Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell*, 11: 1153–1163.

Hartley-Whitaker J, Ainsworth G, Vooijs R, Ten Bookum W M, Schat H, Meharg A A, 2001. Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiol*, 126: 299–306.

Ma L Q, Komar K M, Tu C, Zhang W H, Cai Y, Kennelley E D, 2001. A fern that hyperaccumulates arsenic. *Nature*, 409: 579–579.

Murashige T, Skoog F, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 15: 473–497.

Poynton C Y, Jianwei W, Blaylock H M J, Kochian L V, Elless M P, 2004. Mechanisms of arsenic hyperaccumulation in *Pteris* species: root As influx and translocation. *Planta*, 219: 1080–1088.

Reina S V, Esteban E, Goldsbrough P, 2005. Arsenate-induced phytochelatins in white lupin: influence of phosphate status. *Physiologia Plantarum*, 124: 41–49.

Schmöger M E, Oven M, Grill E, 2000, Detoxication of arsenic by phytochelatins in plants. *Plant Physiol*, 122: 793–801.

Silver S, 1996. Bacterial resistances to toxic metal ions – A review. *Gene*, 179: 9–19.

Tu S, Ma L Q, 2003. Interactive effects of pH, arsenic and phosphorus on uptake As and P and growth of the arsenic hyper accumulator *Pteris vittata* L. under hydroponic conditions. *Environmental and Experimental Botany*, 50: 243–251.

Wang J, Zhao F J, Meharg A A, Raab A, Feldmann J, McGrath S P, 2002. Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. *Plant Physiol*, 130: 1552–1561.

Zhang W, Cai Y, Downum K R, Ma L Q, 2004 Thiol synthesis and arsenic hyperaccumulation in *Pteris vittata* (Chinese brake fern). *Environmental Pollution*, 131: 337–345.