Environmental Pollution 165 (2012) 18–24

Contents lists available at SciVerse ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Phytochelatins play a key role in arsenic accumulation and tolerance in the aquatic macrophyte *Wolffia globosa*

Xin Zhang [a], M. Kalle Uroic [b], Wan-Ying Xie [c], Yong-Guan Zhu [a,c], Bao-Dong Chen [a], Steve P. McGrath [d], Jörg Feldmann [b], Fang-Jie Zhao [d,e,*]

[a] State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China
[b] College of Physical Science, Trace Element Speciation Laboratory (TESLA) Chemistry, University of Aberdeen, Meston Building, Aberdeen AB24 3UE, United Kingdom
[c] Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[d] Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom
[e] College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China

ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form
7 February 2012
Accepted 9 February 2012

Keywords:
Arsenic
Arsenic tolerance
Arsenic speciation
Phytochelatins
Wolffia globosa

ABSTRACT

The rootless duckweed *Wolffia globosa* can accumulate and tolerate relatively large amounts of arsenic (As); however, the underlying mechanisms were unknown. *W. globosa* was exposed to different concentrations of arsenate with or without L-buthionine sulphoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase. Free thiol compounds and As(III)–thiol complexes were identified and quantified using HPLC – high resolution ICP-MS – accurate mass ESI-MS. Without BSO, 74% of the As accumulated in the duckweed was complexed with phytochelatins (PCs), with As(III)–PC_4 and As(III)–PC_3 being the main species. BSO was taken up by the duckweed and partly deaminated. The BSO treatment completely suppressed the synthesis of PCs and the formation of As(III)–PC complexes, and also inhibited the reduction of arsenate to arsenite. BSO markedly decreased both As accumulation and As tolerance in *W. globosa*. The results demonstrate an important role of PCs in detoxifying As and enabling As accumulation in *W. globosa*.

1. Introduction

Arsenic (As) is a class-one carcinogen (National Research Council, 2001). Arsenic contamination in the environment is widespread due to both geogenic and anthropogenic activities. Globally 150 million people are exposed to unsafe levels of As in the drinking water (Brammer and Ravenscroft, 2009). In addition to drinking water, food, especially rice, is also an important source of inorganic As in the diet of some populations (Meharg et al., 2009; Zhu et al., 2008). The common practice of irrigating paddy rice with As-contaminated groundwater in south and southeast Asia may lead to further elevation of As in the food chain (Dittmar et al., 2010; Meharg and Rahman, 2003). Mitigation of As contamination in the environment and in the food chain requires a better understanding of the As biogeochemcal cycle, including the processes of As accumulation and detoxification in the biota (Zhao et al., 2010b).

In aquatic environments or paddy fields, aquatic plants with a high capacity to accumulate As may be used to clean up the contaminant in the water (Rahman and Hasegawa, 2011). Arsenic accumulation has been investigated in a number of aquatic macrophytes (Rahman and Hasegawa, 2011; Robinson et al., 2006; Srivastava et al., 2007; Zhang et al., 2008). *W. globosa* L., a rootless duckweed widely distributed in ponds and paddy fields, was found to be a strong accumulator of As with a relatively high tolerance to the metalloid (Zhang et al., 2009); both of these characteristics are prerequisite for efficient phytoremediation. However, the mechanism of As tolerance in this macrophyte was unknown.

The mechanisms of As tolerance have been extensively studied in terrestrial plants (reviewed by Zhao et al., 2009). Plants are able to take up both arsenate (As(V)) and arsenite (As(III)) via phosphate transporters and some aquaporin channels, respectively. Both forms of As are toxic to cellular metabolism; arsenate interferes with phosphate metabolism such as phosphorylation, whilst arsenite can inactivate enzymes by binding to the sulphydryl groups of the proteins (Hughes, 2002; Ullrich-Eberius et al., 1989). Detoxification of As may involve reduction of arsenate to arsenite, efflux of arsenite to the external medium, complexation of arsenite by thiol-rich peptides and compartmentation of As(III)–thiol complexes in the vacuoles (Zhao et al., 2009). There is strong evidence that phytochelatins (PCs) play a crucial role in arsenite detoxification in

* Corresponding author.
E-mail addresses: Fangjie.Zhao@njau.edu.cn, Fangjie.Zhao@rothamsted.ac.uk (F.-J. Zhao).

0269-7491/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.envpol.2012.02.009

terrestrial plants. PC-deficient mutants of *Arabidopsis thaliana* were found to be much more sensitive to As than the wild-type plants (Ha et al., 1999; Liu et al., 2010). Over-expression of PC synthase enhanced As tolerance in the transgenic plants (Gasic and Korban, 2007; Guo et al., 2008; Li et al., 2004). In contrast, use of L-buthionine sulphoximine (BSO) to inhibit the synthesis of glutathione (GSH) and PCs resulted in greatly increased sensitivity to As in a number of plant species (Schat et al., 2002; Schmöger et al., 2000). Recent studies have also identified a number of As(III)–thiol complexes in plant extracts (Bluemlein et al., 2008; Liu et al., 2010; Raab et al., 2007). Furthermore, two ABC transporters able to mediate the transport of As(III)–PCs into the vacuoles have recently been identified in *Arabidopsis* (Song et al., 2010). Unlike As non-hyperaccumulating plants, As hyperaccumulating ferns (e.g., *Pteris vittata*) appear to employ a PC-independent mechanism of As detoxification, with only a very small proportion of the As taken up being complexed with PCs (Raab et al., 2004; Webb et al., 2003; Zhang et al., 2004; Zhao et al., 2003). In these plants, arsenate is reduced to arsenite, which is sequestered in the vacuoles mostly in the non-complexed form (Lombi et al., 2002; Pickering et al., 2006), probably via the tonoplast arsenite transporter *Pv*ACR3 (Indriolo et al., 2010). The mechanisms of As tolerance in aquatic macrophytes are not well understood. Studies with the Esthwaite waterweed *Hydrilla verticillata* showed that As exposure was accompanied by increased PC synthesis and that increasing the sulphur supply enhanced As tolerance as well as As accumulation (Srivastava and D'Souza, 2009; Srivastava et al., 2007).

The objectives of this study were to identify and quantify As(III)–thiol complexes and to investigate the role of thiol-rich peptides in As tolerance and accumulation in *W. globosa*.

2. Materials and methods

2.1. Plant culture

W. globosa L. was collected from Wuhan, Hubei province, China, and maintained in a half-strength Hoagland nutrient solution. Progeny from this original stock was used in the present study. Plants were grown in the half-strength Hoagland solution for 3 weeks before being used in experiments. The composition of the nutrient solution was as follows: 2 mM $CaNO_3$, 3 mM KNO_3, 1 mM $MgSO_4$, 0.5 mM $NH_4H_2PO_4$, 100 μM Fe–EDTA, 46 μM H_3BO_3, 9 μM $MnCl_2$, 0.75 μM $ZnSO_4$, 0.35 μM $CuSO_4$ and 0.55 μM Na_2MoO_4 (pH adjusted to 6.0 with KOH or HCl solutions). Nutrient solution was renewed twice every week. The hydroponic culture and all experiments were conducted inside a controlled-environment growth chamber with the following conditions: 14 h light period d^{-1} with a light intensity of *c*. 280 μmol m^{-2} s^{-1}, 25 : 20 °C day : night temperatures, and 70% relative humidity.

2.2. Arsenate accumulation, speciation and tolerance in W. globosa

After preculture for 3 weeks, *W. globosa* plants were transferred to 28 pots (80 ml, 3 g plant material per pot) and treated with 500 μM L-buthionine sulphoximine (BSO) for 5 d; another group of 28 pots not treated with BSO served as the control. Thereafter, plants in both groups were exposed to 0, 1, 5, 10, 30, 50, 100 μM arsenate (Na_3AsO_4) for 5 d, with or without 500 μM BSO. Each As and BSO treatment was replicated in four pots. After 5 day exposure, plants were harvested, washed with deionized water, and then immersed in an ice-cold desorption solution containing 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$, and 5 mM MES (pH 5.5) for 15 min to remove apoplastic As. Plants were blotted dry, weighed, frozen in liquid nitrogen and freeze-dried. The concentrations of total As and As speciation were determined as described below.

2.3. Arsenate uptake and arsenite efflux

Four replicates of 3 g *W. globosa* were treated with 200 ml 500 μM BSO for 3 d; another group was not treated with BSO. Three days later, plants were exposed to 5 μM arsenate, with or without 500 μM BSO. Aliquots of 0.5 ml nutrient solution were collected at 1, 5, 10 and 24 h, diluted with 4.5 ml phosphate-buffered solution (PBS, 2 mM NaH_2PO_4 and 0.2 mM Na_2–EDTA, pH 6.0), and filtered through 0.45 μm before analysis of As speciation. Arsenate uptake and arsenite efflux were calculated from the decrease of arsenate and the appearance of arsenite in the nutrient solution, respectively. After 24 h, plants were harvested for total As and As speciation analysis as described below. To evaluate the potential role of microorganisms versus *W. globosa* in mediating arsenate reduction in the uptake solution, arsenate (5 μM) was added to three solutions: fresh nutrient solution without *W. globosa*, fresh nutrient solution with 3 g *W. globosa*, and old nutrient solution that had been used to grow *W. globosa* for 3 days and with the duckweed subsequently removed. Each treatment was replicated in 4 pots. Arsenic speciation in the nutrient solutions was monitored at 1, 5, 10 and 24 h.

2.4. Arsenite complexation with thiol compounds

W. globosa (3 g) was incubated in 50 ml nutrient solution with the phosphate concentration being lowered to 0.05 mM. Four replicates were treated with 500 μM BSO for 3 d and another group was not treated with BSO. Three days later, plants were exposed to 10 μM arsenate with or without 500 μM BSO for 4 days. Plants were harvested and As(III)–thiol complexes and free thiol compounds determined as described below.

2.5. Analysis of total As in plant tissues

Ground plant samples were digested in 5 ml high-purity $HNO_3/HClO_4$ (87/13, v/v). Total As concentrations in the digests were determined by ICP-MS (Agilent 7500ce) operating in the helium gas mode to remove possible interference of $^{40}Ar^{35}Cl$ on *m*/*z* 75.

2.6. Analysis of As speciation in plant tissues

Samples were ground in liquid nitrogen to fine powder with a mortar and pestle. Samples (0.1 g) were extracted with 10 ml PBS solution for 1 h under sonication. The extract was filtered through Whatman NO. 42 filter paper and then a 0.2 μm filter. The efficiency of As extraction by PBS was approximately 60%. Arsenic speciation was determined by anion-exchange HPLC-ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7500ce; Agilent Technologies). Chromatographic columns consisted of a Hamilton precolumn (11.2 mm, 12–20 mm) and a Hamilton PRP-X100 10 μm anion-exchange column (150 × 4.1 mm). As species [As(III), As(V), DMA, and MMA] were separated with a mobile phase of 4.4 mM $NH_4H_2PO_4$ and 4.4 mM NH_4NO_3, 0.2 mM EDTA and 2.5% (v/v) methanol (pH 6.2), run isocratically at 0.65 mL min^{-1}. Germanium (Ge) as an internal standard was mixed continuously with the post-column solution through a peristaltic pump. Signals at *m*/*z* 75 (As) and 72 (Ge) were collected with a dwell time of 300 and 100 ms, respectively. Possible polyatomic interference of $^{40}Ar^{35}Cl$ on *m*/*z* 75 was removed by the Agilent Octopole Reaction System operating in the helium gas mode. The As signal was normalized using the Ge signal to correct any signal drift during the analysis. As species in the samples were identified by comparisons with the retention times of standard compounds and quantified by external calibration curves with peak areas. Analysis of As species was carried out immediately following sample collection or extraction.

2.7. Analysis of thiol compounds and As(III)–thiol complexes

For the analysis of As(III)–thiol complexes and free thiols, duckweed samples were ground in liquid nitrogen to fine powder with a mortar and pestle. Samples (1 g) were then transferred to 15-ml Grenier tubes, to which 5 ml ice-cold 1% formic acid was added. Samples were extracted at 4 °C for 1 h, with intermittent shaking by hand. Samples were subsequently centrifuged at 890*g* for 3 min after extraction. Approximately 1 ml of supernatant was then transferred to an Eppendorf vial, where the supernatant was further centrifuged at 7550 *g* for 5 min and used for speciation analysis. Analysis of As(III)–thiol complexes and free thiol compounds was carried out using HPLC coupled with high resolution ICP-MS (Element 2; Thermo Fisher Scientific) and high resolution ESI-MS (LTQ Orbitrap Discovery; Thermo Fisher Scientific), as described previously (Bluemlein et al., 2009; Liu et al., 2010). Separation was performed on a C_{18} reverse-phase column (Eclipse XDB-C18, 5 μm), using a water-methanol gradient. Starting from 100% water, methanol was added to the eluent at the rate of 1% per min over the first 20 min. The eluent was held at 80% water/20% methanol for a further 10 min, followed by switching back to 100% water. Total chromatographic run time was 30 min. For the ICP-MS analysis, As and S were measured on *m*/*z* 75 and 32, respectively. Germanium was added post-column as internal standard for the ICP-MS, and its signal was measured on *m*/*z* 72. For As and S quantification, a blank run was done for response factor (Rf) determination with a post-column addition of As, S and Ge. To account for the change in the sensitivity due to the methanol gradient, each chromatographic run was divided by the response factor run. These normalized chromatograms were used for peak integration with PeakFit and subsequent quantification. According to a previous study with sunflower plants (Raab et al., 2005), the method used extracted 70–90% of the total As and the column recovery was appropriately 90%. The As speciation determined by the method used was found to be in good agreement with that determined by non-destructive X-ray absorption spectrometry (Bluemlein et al., 2008).

2.8. Data analysis

All data were subjected to analysis of variance (ANOVA) using windows-based SPSS 13.0.