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A significant positive correlation between endogenous *trans*-zeatin content and total arsenic in arsenic hyperaccumulator *Pteris cretica* var. *nervosa*

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ABSTRACT

A pot experiment was conducted to compare the content of endogenous *trans*-zeatin (Z), plant arsenic (As) uptake and physiological indices in the fronds of As-hyperaccumulator (*Pteris cretica* var. *nervosa*) and non-hyperaccumulator (*Pteris ensiformis*). Furthermore, a stepwise regression method was used to study the relationship among determined indices, and the time-course effect of main indices was also investigated under 100 mg/kg As stress with time extension. In the 100–200 mg/kg As treatments, plant height showed no significant difference and endogenous Z content significantly increased in *P. cretica* var. *nervosa* compared to the control, but a significant decrease of height and endogenous Z was observed in *P. ensiformis*. The concentrations of As (III) and As (V) increased significantly in the fronds of two plants, but this increase was much higher in *P. cretica* var. *nervosa*. Compared to the control, the contents of chlorophyll and soluble protein were significantly increased in *P. cretica* var. *nervosa* but decreased in *P. ensiformis* in the 200 mg/kg As treatment, respectively. A significant positive correlation was found between the contents of endogenous Z and total As in *P. cretica* var. *nervosa*, but such a correlation was not found in *P. ensiformis*. Additionally, in the time-course effect experiment, a peak value of each index was appeared in the 43rd day in two plants, except for chlorophyll in *P. ensiformis*, but this value was significantly higher in *P. cretica* var. *nervosa* than that in *P. ensiformis*. In conclusion, a higher endogenous Z content contributed to As accumulation of *P. cretica* var. *nervosa* under As stress.

1. Introduction

In recent years, arsenic (As) pollution occurs frequently in soil and water due to anthropogenic discharge, such as the use of herbicides, insecticides, pesticide, livestock dips and wood preservatives (Fayiga et al., 2005). Arsenic pollution is of increasing environmental concern owing to its risk to plants, animals and human health. Arsenic is an unnecessary element for plant growth, and will result in plant toxicity if it is presented at a high level. For this reason, it is imperative to decontaminate As in soil and water. With the discovery of *Pteris vittata*, a well-known As hyperaccumulator (Ma et al., 2001), phytoremediation of As-polluted soil has made considerable progress. Today, at least 21 As hyperaccumulators have been identified, and most of them belong to *Pteris* genus of Pteridaceae, except for *Pityrogramma calomelanos*, which belongs to Hemionitidaceae (Xie et al., 2009). Additionally, no difference is shown in the ability of As-hyperaccumulation among different populations of *P. vittata* (Zhao et al., 2002). Although *Pteris ensiformis* (Singh et al., 2006) and *Pteris semipinnata* (Wang et al., 2007) also belong to *Pteris* genus, but they show no evidence of As-hyperaccumulation. Plants with different As hyperaccumulative ability are excellent experimental materials for the comparative study of As tolerant, hyperaccumulative and detoxificative mechanisms in plants.

In general, some previous studies regarding As hyperaccumulators mainly focused on As uptake with an excessive amount. Recently, Han et al. (2017) found that arsenite oxidase genes in the rhizosphere soil of *P. vittata* are 50 times more abundant than the arsenate reductase genes, which may contribute to the phytoremediation of As-contaminated soil. However, the reasons why As hyperaccumulators can maintain good growth after accumulating high concentrations of As are often neglected. Plant hormones play an important role in the response to heavy metal stress and regulation of plant growth (Atici et al., 2003). Cytokinin (CTK), a common plant hormone, has extensive biological effects on plants, such as stimulating cell division, delaying leaf senescence, promoting plant cotyledon expansion and chlorophyll biosynthesis (Bajguz and Piotrowska, 2009) and redistributing of potassium etc. (Tu and Ma, 2005), although its content in plants is

Abbreviations: As, arsenic; BHT, butylated hydroxytoluene; CAT, catalase; Chl, chlorophyll; CKXs, cytokinin oxidase; CRE1, CTK response 1; CTK, cytokinin; DEAE, donediethylaminoethanol; DMA, dimethylated arsenic; MDA, malondialdehyde; PCs, phytochelatins; POD, peroxidase; PVPP, polyvinylpolyrrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase; Z, *trans*-zeatin

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very low.

CTK has at least dual roles in plant metal uptake and tolerance under heavy metal stress. On the one hand, CTK can increase the heavy metal accumulation (Pb and Zn) in shoots of *Helianthus annuus* by increasing its transpiration rate (Tassi et al., 2008). Furthermore, Wang et al. (2015) found that the concentration of As in shoots of maize seedlings is significantly increased with the addition of 1.5 mg/L kinetin (one type of CTKs, KT). In addition, the concentration of Cd in the roots of maize seedlings is significantly increased by 7.5 mg/L 6-benzylaminopurine (one type of CTKs, 6-BA) and 70 mg/L KT, respectively, and this increase is also shown in its shoots by adding 20 mg/L 6-BA or 100 mg/L KT (Xu et al., 2010). On the other hand, plants display a strong tolerance to cope with the increasing concentrations of heavy metals induced by CTK addition. In the study mentioned above, the catalase (CAT) and peroxidase (POD) activities are also significantly increased in the leaves of maize seedlings by 1.5 mg/L KT under As stress, and the malondialdehyde (MDA, a peroxidation product of cell membrane lipids) content is significantly reduced (Wang et al., 2015). Similar results are also reported by Xu et al.(2010), who further found the content of proline in the leaves of Cd-stressed maize seedlings is significantly increased with 6-BA or KT application. These results show that the toxic effects of heavy metal (loids) on plants can be alleviated with CTK addition by reducing reactive oxygen species (ROS) generation or keeping osmotic pressure of cells. Besides this, CTK promotes the accumulation of heavy metals by increasing metallothionein-like gene expression and the contents of metal transporter proteins (Thomas et al., 2005).

For endogenous CTK, main studies focus on drought (Rivero et al., 2007) and salt stress (Nishiyama et al., 2011), but only limited information is available under heavy metal stress. A decreasing content of endogenous CTK was observed by Cd in short (3 h) and long-termed (14 d) treatment in wheat seedlings (Veselov et al., 2003). Hayward et al. (2013) also found that the content of endogenous CTK is remarkably declined in *Deschampsia cespitosa* in the 5 μg/mL Cd treatment group, but no significant change is noted under Ni stress. Interestingly, Pb increases endogenous *trans*-zeatin (one type of CTKs, Z) content of chickpea seedlings, which may result from the stimulation of Pb on the Z *de novo* synthesis (Atici et al., 2005). However, the variation of endogenous CTK in *P. cretica* var. *nervosa* (As hyperaccumulator) and *P. ensiformis* (non-As hyperaccumulator) under As stress is unclear.

There are some indices to evaluate the toxicity responses of plants to As stress, such as biomass, photosynthesis, soluble protein and soluble sugar etc.. It is undoubtedly that CTKs regulate plant adaptation to adverse environment via remaining plant biomass, increasing stomatal apertures, transpiration (Ha et al., 2012) and photosynthetic activity by increasing the electron donation capacity of photosynthesis system II (PSII) (Shao et al., 2010). Photosynthesis is characterized by converting sunlight into chemical energy, in which the chlorophyll (Chl) plays an indispensable role. Arsenic inhibits the Chl biosynthesis in maize (Jain and Gadre, 1997), and the maximal photochemical efficiency (Fv/Fm) and actual quantum efficiency (F_{PSII}) are significantly decreased by 100 mg/kg As in *P. vittata* (Wang et al., 2012). In addition, most of soluble proteins in plants are enzymes which are involved in metabolic reactions, so it is an important index to reflect plant total metabolism.

In the present study, the content of Z was measured in the fronds of As hyperaccumulator *P. cretica* var. *nervosa* and non-hyperaccumulator *P. ensiformis* under different concentration of As (V) stress by a pot experiment. In addition, plant biomass, height, Chl and soluble protein contents were also determined to evaluate plant growth status under As stress. Moreover, the variation of these indices was further analyzed with culture time extension in the 100 mg/kg As (V) treatment. This work will explore the relationship among contents of endogenous Z, Chl, soluble protein and As accumulation in the fronds of the two *Pteris* plants under As stress.

2. Materials and methods

2.1. Soil and plant preparation

Soil was sampled from the campus of Kunming University of Science and Technology (KUST). Soil samples were air dried and passed through 5 mm nylon sieve. Then, soil, sand and humus were mixed with the ratio of 2:1:1, respectively. The soil was artificially polluted by As(V) at four levels (0, 50, 100 and 200 mg/kg). Arsenic was added as $Na_3AsO_4{\cdot}12H_2O$ and the As concentration was calculated as pure As. Soil N, P (P_2O_5) and K (K_2O) were also added with the ratio of 0.15: 0.10: 0.15 g/kg soil and mixed thoroughly for 6 weeks. Soil pH value was determined by potentiometric (soil-water ratio, 1:2.5), organic matter content by potassium dichromate method-thermodilution, total P by molybdenum antimony colorimetric method, available K by 2 mol/L cold HNO_3 extraction-flame atomic absorption spectrometry (FAAS, Model AA240FS, Varian, USA), total N by semimicro Kjeldahl, organic C by potassium dichromate method-external heating, and cation exchange capacity (CEC) by ammonium acetate method. The concentration of Si in soil was determined by Na_2CO_3 fusion-weight method. The soil was digested with aqua regia-$HClO_4$ (4:1, v/v) for heavy metal (loid) analysis. The concentrations of Pb, Zn, Cu and Cd in digestion were determined by FAAS (Model AA240FS, Varian, USA), As by stomic fluorescence spectrometry (AFS, AF-610D, Beijing Rayleigh Analytical Instrument Co., Ltd, China), and Al by inductively coupled plasma spectroscopy (ICP-OES, Agilent, USA). The concrete methods for the soil physico-chemical properties above mentioned were referred by Page et al. (1982) and Bao (2000). The soil pH was 7.63 ± 0.10. The contents of organic matter, total N, total P, available K in soil were 42.5 ± 2.8, 1.5 ± 0.5, 2.1 ± 0.4 and 0.7 ± 0.1 g/kg, respectively. The concentrations of Pb, Zn and Cu in soil were 0.68 ± 0.03, 9.0 ± 1.2 and 35 ± 4 mg/kg, respectively, but As and Cd were below the limit of detection (As: 0.011 mg/kg, Cd: 0.006 mg/kg). The cation exchange content (CEC), C/N and Si/Al in soil were 28.2 ± 1.4 cmol(+)/kg, 24.6 ± 1.6 and 3.00 ± 0.01, respectively.

Twenty four small plastic pots (9.5 cm × 16 cm, 12 pots for one plant species) were prepared, and each was filled with 1 kg mixed soil. In addition, 6 big plastic pots (18 cm × 24 cm, 3 pots for one plant species) were prepared for the time-course experiment, and each was filled with 20 kg mixed soils containing 100 mg/kg As. Each treatment was replicated three times.

The seedlings of *P. cretica* var. *nervosa* and *P. ensiformis* were sampled from the campus of KUST and Hekou county, Yunnan Province, China, respectively. They were taken back to the lab and planted in clean soil for adaptive culture about 2 months. The plants with good growth and similar size were chosen for pot experiment. Plants were grown in a greenhouse under natural light condition, with night/day temperature varying from 8 °C to 22 °C. Plants were generally watered twice a week or when necessary. No additional fertilizers were applied to the soils during the experimental period. Plants were harvested at day 49 after plant transplanting, then washed thoroughly with tap water and their height and dry weight were recorded. For time-course experiment, the plants were collected every 6 days (1, 7, 13, 19, 25, 31, 37, 43, 49) to measure the related indices.

2.2. Measurement of total As and its speciation in fronds

In order to determine As concentration in fronds of plants, part of plant fronds was taken out and washed with 1% hydrochloric acid (HCl), then rinsed with tap and deionized water to remove bound As on frond surface. The plant tissues were dried in a hot air oven at 105 °C for 30 min and dried to constant weight at 70 °C. The dried plant materials were ground to a fine powder. About 0.2 g dried sample was weighted and digested by HNO_3-H_2O_2 below 130 °C to avoid As volatilization (Wang et al., 2007), and total As concentration was determined by atomic fluorescence spectrometer (AF-610D, Beijing