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Glutathione plays a role in protecting leaves of *Salvinia minima* from Pb^{2+} damage associated with changes in the expression of SmGS genes and increased activity of GS

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ABSTRACT

The relationship between accumulation of Pb^{2+} and changes in GSH biosynthesis was analyzed in both leaves and roots of the Pb^{2+}-hyperaccumulator aquatic fern *Salvinia minima*, after exposure to 40 μM $Pb(NO_3)_2$. Lead accumulation in both tissues increased the accumulation of GSH, increased the enzymatic activity of glutathione synthase (GS), and caused changes in the expression levels of *Sm*GS genes in both tissues. The damage caused by Pb on plant performance, was evaluated by the changes in the content of pigments, particularly on the carotenoids content. Lead accumulation caused more damage in roots than in leaves as indicated by the decrease on their carotenoids content. It is interesting that in leaves, the concentration of GSH, the activity of GS and the expression levels of *Sm*GS gene were all higher than in roots. These results, together with our previous finding that roots accumulated more phytochelatins than did leaves of *S. minima* plants exposed to similar concentrations of lead (Estrella et al., 2009), suggest that the Pb-hyperaccumulator aquatic fern, *S. minima*, displays a coordinated and differential response to Pb^{2+} at leaves and roots, where GSH may play an important role in protecting leaves from the detrimental effects of lead, perhaps by counteracting the effect of free radicals.

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1. Introduction

Gluthatione (γ-L-glutamyl-L-cysteine-L-glycine; GSH) is a tripeptide distributed extensively in the majority of cells; it is a low molecular weight non-protein compound with antioxidative properties. GSH is synthesized in two steps. The first step involves the formation of γ-L-glutamyl-L-cysteine (γ-EC) from L-glutamate and L-cysteine, this step is catalyzed by the enzyme γ-glutamylcysteine synthetase (γ-EC, EC 6.3.2.2.) while the second step is the conjugation of γ- L-glutamyl-L-cysteine with glycine; this step is catalyzed by the enzyme glutathione synthase (GS, EC 6.3.2.3.) (Meister, 1995). The biosynthesis of GSH involves sulfur assimilation in the form of sulphate (Leustek et al., 2000). Glutathione participates in a variety of activities such as signal transduction pathways and cellular defense (Noctor and Foyer, 1998), as the substrate of GSH-S-transferase, which catalyzes the conjugation of GSH with xenobiotic substances, such as weed killers (Marrs, 1996). In addition, GSH actively participates in the redox balance preventing the accumulation of free radicals and hence reducing oxidative stress. Evidence exists for the participation of GSH in stress caused by exposure to heavy metals. In cell cultures of tomato and tobacco, GSH concentration increased significantly in the presence of Cd^{2+} (Chen and Goldsborough, 1994); a similar behavior was observed in roots of maize (Rüegsegger and Brunold, 1992). On the other hand, changes in the expression of the gene glutathione synthase (GS) have been documented in *Arabidopsis thaliana* and *Schizosaccharomyces pombe* in response to Cd^{2+} (Harada et al., 2002; Kim et al., 2003). Glutathione is also the immediate precursor of phytochelatins (PC). Accumulation of PC is considered to be an important mechanism of heavy metal detoxification in terrestrial plants (Xiang et al., 2001) and aquatic plants such as *Salvinia minima*, where the presence of Pb^{2+} increases the production of this cysteine rich polypeptide (Estrella et al., 2009).

S. minima is an aquatic fern capable of removing and accumulating heavy metals which exhibits high growth rate and elevated biomass production and is easy to handle and collect from water body surfaces (Estrella et al., 2009). Each unit consists of three fronds, two floating fronds (leaf-like fronds) and a submerged modified frond (root-like frond). The abaxial surface of the floating fronds has abundant structures similar to absorbent hairs, which are in direct contact with water. Leaf-like fronds are referred to in this paper as "leaves", while root-like fronds are referred to in this paper as "roots". Under controlled culture conditions, *S. minima* removes 82% of Cd^{2+} (10.9 ppm) and 97% of Pb^{2+} (9.7 ppm)

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added to the culture, and, in fact, it has been described as a hyperaccumulator for Pb^{2+} (Olguín et al., 2002).

Therefore, in the present paper, an attempt is made to establish whether a relationship exists between the capacity of *S. minima* plants to accumulate and tolerate Pb^{2+}, and their capacity to increase their GSH production, GS activity, and/or modify the expression of *S. minima* gene encoding GS (*SmGS*). This study should contribute to an increased understanding of the possible participation of GSH in a part of a lead detoxification mechanism.

2. Materials and methods

2.1. S. minima growing culture

Plants of the aquatic fern *S. minima Baker* (Salviniaceae) ecotype Yucatán, were cultivated in hydroponics conditions in a modified Hoagland's solution (Hoffmann et al., 2004), at 25 ± 2 °C, in a greenhouse with a photon flux density that varied from 25 to 120 μmol m^{-2} s^{-1}, a relative humidity of 70 ± 3.6%, and with a natural light photoperiod of 12 h.

2.2. Determination of internal Pb^{2+} concentrations in tissues

From our previous work, we were expecting early responses of *S. minima* to lead, so samples were taken every 3 h during the first 12 h (0, 3, 6, 9, 12), later we took samples at day 1 and 2 (24, 48 h) with the objective of evaluating later responses, while the last point was taken to define if the trends found at earlier times would remain after 5 days of exposure. At each sampling time, plants from each treatment were washed with 10 mM EDTA pH 8.0 followed by a rinse with de-ionized water, to remove external metal ions. Pb^{2+} was quantified according to Hoffmann et al. (2004).

2.3. Determination of Pb^{2+} removal

The quantification of Pb^{2+} in the solution was made on 3 mL of medium in which *S. minima* plants were exposed to 40 μM of $Pb(NO_3)_2$ for 0, 3, 6, 9, 12, 24, 48 and 120 h and subsequently analyzed in an atomic emission spectrometer inductively coupled to plasma (ICP-IOES 400 PERKIN ELMER). The detection of Pb^{2+} was carried out by readings at 220 and 353 nm using 1% HNO_3 as blank and spectrometer calibration standards of 100.0, 10.0, 1.0 and 0.1 ppm of Pb^{2+} (Merck) (Hoffmann et al., 2004).

2.4. Quantification of chlorophyll concentration

The chlorophyll content determination was performed according to the method described by Dere et al. (1998). One gram of fresh tissue was crushed in a mortar, adding 20 mL of acetone 99.9% (Sigma) for extracting pigments. Subsequently, in order to separate the cellular tissue of the pigments, the extraction solution was filtered using a Whatman # 1 filter. The filtrate was recovered and brought up to 50 mL with acetone. Finally, spectrophotometric readings were made at absorbances of 662, 645 and 470 nm with a visible light lamp using a spectrophotometer DU 650 (Beckman Coulter). The concentration of each pigment was reported as mg g dw^{-1} and calculated as:

$$Ca = 11.75\,(A662) - 2.350(A645).$$
$$Cb = 18.61\,(A645) - 3.960\,(A662).$$
$$Ct = 1000\,(A470) - 2.270\,(Ca) - 81.4\,(Cb)/227.$$

where *Ca*, Chlorophyll *a*; *Cb*, chlorophyll *b*; *Ct*, total carotenoids; A662, A645 and A470 nm, readings of absorbance's at these wavelengths.

2.5. Quantification of gluthatione (GSH)

Extraction of GSH was carried out by a modification of the method of Grill et al. (1991), using 250 mg of freeze-dried tissue (roots or leaves). Freeze-dried material was ground in a mortar in 1.5 mL of 50 mM Tris (Sigma) pH 7.0, 100 mM ascorbic acid (Sigma), 1 mM DTT (Sigma). This mixture was homogenized for 1 min. The samples were centrifuged at 15 000 × *g* for 30 min at 4 °C. An aliquot of supernatant (350 μL) was derivatized with 100 μL 0.5 M HEPES pH 8.0, 0.5 μL 0.5 M EGTA, pH 8.0 and 1 μL 100 mM monobromobimane (Fluka, St. Louis, MO, USA). The tubes were incubated at 30 °C for 12 h in the dark. Finally, the reaction was stopped by adding 50 μL of 30% trichloroacetic acid and shaking the tubes for 5 min. The samples were filtrated through Millex filters of 0.45 μm pore diameter (Millipore, Jaffrey, NH, USA) and injected (20 μL) into a high performance liquid chromatography apparatus (HPLC-200, USA) equipped with a fluorescence detector. The metabolites were separated on a C18 reverse phase column (Phenomenex 6 nm, 5 mm, and 3.9 mm × 100 mm) and using a linear gradient of methanol–acetic acid pH 3.9. The L-cysteine (Sigma, USA) and the glutathione (Sigma, USA) were used for identification and curve calibration.

2.6. Quantification of proteins

Total protein content was quantified using the method of Bradford (1976). A calibration curve was constructed using dilutions from an initial solution of 1 mg/mL of bovine serum albumin (BSA, Sigma).

2.7. Activity of glutathione synthetase (GS)

The activity of glutathione synthetase (GS) was determined through the modified method of Kim et al. (2003). The assay is based on measuring the formation of adenosine diphosphate (ADP) spectroscopically. The reaction mixture contained 100 mM Tris–HCl pH 7.8, 100 mM potassium chloride (KCl, Sigma), 5 mM adenosine triphosphate (ATP, Sigma), 50 mM magnesium chloride ($MgCl_2$, Sigma), 10 mM glycine, 5 mM γ-L-glutamyl-L-cysteine (γ-EC, Sigma), 0.4 mM phosphoenolpyruvate (Sigma), 0.2 mM nicotinamide adenine dinucleotide (NADH, Sigma), 5 μL of pyruvate kinase (5 U/μL, Sigma), 2.5 mM lactate rabbit muscle type II (Sigma). The reaction was initiated by adding 400 μg total protein and incubated for 2.5–30 min at 37 °C. The amount of ADP was calculated by the change in the readings obtained at an absorbance of 340 nm with 100 μL (1 U/μL) of lactate dehydrogenase.

2.8. Isolation of RNA and semi-quantitative PCR analysis of SmGS

Total RNA was isolated from roots and leaves using CTAB and PVP in the extraction buffer and LiCl for RNA precipitation, according to the method described by Gasic et al. (2004). The purity of RNA samples and their concentrations were determined spectrophotometrically, where the A260/A280 ratios obtained were between 1.8 and 2.0. Five micrograms of total RNA were reverse transcribed into cDNA by using 1 μL Superscript RT II (50 U/μL) (Invitrogen, CA, USA) and incubated for 90 min at 42 °C.

The analysis of the expression of glutathione synthetase was performed by PCR using the following amplification program: 95 °C for 2 min, 49 °C for 2 min, 72 °C for 1 min in 38 cycles. The primers utilized to carry out the amplification of the fragment of *SmGS* were 5′-CCAGACAGTTGCTGTGGTGT-3′ (Forward, Integrated DNA Technologies) and 5′-ACCATCACGGACCAGAAAAG-3′ (Reverse, Integrated DNA Technologies). The PCR contained 2 μg of ADNc 1.0 pmol of primers (forward and reverse), 1 μL of 40 mM $MgCl_2$ (Invitrogen) and 25 μL of PCR supermix High fidelity

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