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Genes coding for transporters showed a rapid and sharp increase in their expression in response to lead, in the aquatic fern (*Salvinia minima* Baker)

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ABSTRACT

Salvinia minima was assessed for its ability to accumulate lead (Pb) by exposing it to concentrations of 40 μM Pb $(NO_3)_2$ during 24 h. At the same time, the expression levels were quantified, of four genes coding for transporters: *SmABCC* (ABCC-MRP), *SmATPase* (ATPase-P3A), *SmNhaD* (Type-Na^+/H^+) and *SmABCG* (ABCG-WBC). In the absence of lead, *S. minima* had very low expression of those genes, when plants were exposed to the metal however, those genes showed a rapid (in just three hours or less) and sharp increase (up to 60 times) in their expression, particularly the *SmNhaD* (Type-Na^+/H^+) gene. This sharp increase in expression levels of the genes studied, occurred at the same time that the plant accumulated the highest content of lead in its tissues. The first two genes, are apparently implicated in detoxification and lead accumulation mechanisms, while the other two genes are apparently involved in maintaining cell balance (homeostatic control) and membrane integrity. Our results confirmed that *S. minima* is efficient for phytoremediation of water bodies contaminated by lead, as it is efficient in accumulating this metal in its tissues (bioconcentration factor; BCF) values greater than 1000, in short times of exposure. More importantly, our data on the expression profiles of four genes coding for transporters, represent a first sight scenario of the molecular basis for understanding the different mechanism of detoxification, apparently present in this aquatic fern.

1. Introduction

The contamination of heavy metals in aquatic ecosystems represents a global environmental problem that becomes a serious concern in many regions of the world. Among common pollutants that affect plants, lead (Pb) is one of the most toxic and frequently encountered (Cecchi et al., 2008; Grover et al., 2010; Shahid et al., 2011). Pb is a metal commonly used in welding, paints, ammunition, leaded glass and batteries, for this reason, Pb is found in all environments (soils, water, the atmosphere, and living organisms) (Islam et al., 2007; Andra et al., 2009; Punamiya et al., 2010). Different methodologies are currently used for the decontamination of industrial effluents, including electrodialysis, reverse-osmosis, ion exchange, and other methods, but the methodologies used are quite costly and energy intensive, and not sustainable for treating large volumes of wastewater (Miretzky et al., 2004; Malik, 2007; Mishra and Tripathi, 2008).

Phytoremediation is a cost-effective and environmentally friendly methodology, that uses the plants with the potential to accumulate and tolerate heavy metals, (Shi and Cai, 2009). Phytoremediation plants are those that have a high Bioconcentration factor (BCF), which indicates the efficiency of metal- accumulation in phytoremediation trials (Gothberg et al., 2004; Duman et al., 2009; Maldonado-Magaña et al., 2011). Pb is taken by the root system; it may accumulate there, or it may be translocated to aerial plant parts. For most plant species, the majority of the absorbed Pb (approximately 95% or more) is accumulated in the roots, and only a small fraction is translocated to aerial plant parts, as it has been reported in *Vicia faba*, *Pisum sativum*, and in *Phaseolus vulgaris* (Piechalak et al., 2002; Małecka et al., 2008; Shahid et al., 2011), *Nicotiana tabacum,* (Gichner et al., 2008), *Zea mays* (Gupta et al., 2009), and in *Salvinia minima* (Hoffmann et al., 2004).

Salvinia minima is an aquatic fern with the potential to grow rapidly in water bodies, this plant has the capacity to accumulate different metals into tissues such as: Cd (Olguin et al., 2002), Cr (Olguin et al., 2002; Prado et al., 2010), Ni (Fuentes et al., 2014), Pb (Olguin et al., 2002; Hoffmann et al., 2004; Leal-Alvarado et al., 2016). In the case of Pb, *S. minima* plants has shown BCF in the range of 2159–3304 (Olguin

Abbreviations: DW, dry weight; EDTA, ethylene diamine tetra acetic acid; BCF, Bioconcentration factor; TF, Translocation factor; Δ-Pb, Lead delta; REL, Relative expression levels

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et al., 2002; Hoffmann et al., 2004).

Despite this great capacity to uptake lead, previous studies have shown that *S. minima* plants exposed for 12 days to 40 μM $Pb(NO_3)_2$, although they did not present visible effects, they experienced a reduction of 50% in leaf survival, (Hoffmann et al., 2004) and a reduction in various physiological parameters when exposed to the same concentration of lead but at shorter exposure periods of 24 h (Leal-Alvarado et al., 2016).

Among the few studies focused on understanding the molecular basis for the response of *S. minima* to lead, Estrella-Gómez et al., (2009, 2012) studied the role played by phytochelatins and glutathione synthase on the lead detoxification mechanisms (Estrella-Gómez et al., 2009, 2012). However, the molecular basis of the mechanisms involved in the ability to accumulate and tolerate lead, are not yet fully known. The understanding of the cellular and molecular response to lead in *S. minima* is therefore essential to maximize the use of *S. minima* in phytoremediation.

Previous studies have focused on understanding the role of some genes on detoxification mechanisms (glutathione and phytochelatin), when exposed to heavy metals (such as Pb, Cu, Zn) (Małecka et al., 2008; Liu et al., 2009; Estrella-Gómez et al., 2009, 2012; Gupta et al., 2010; Ahmad and Gupta, 2013; Li et al., 2015; Benyó et al., 2016). In addition, some other studies have evaluated the role of different transporters on the plant response to heavy metals induce stress. Transporters such as: ABC transporters, P-type ATPase transporters and Na^+/H^+ antiporters Among the family of ABC transporters, some ABC transporters are involved in various processes and stages of the plants development (Kang et al., 2011), some others are involved in detoxification plants systems in response to heavy metals. Among these, the ATP-dependent tonoplast transporters (ABC transporters) are involved in the exportation of glutathione conjugates from the cytosol into the vacuole, increasing the plants ability to tolerate heavy metals (Bovet et al., 2003, 2005; Gaillard et al., 2008; Wojas et al., 2009). 2) Regarding the P-type ATPase transporters: The P-Type ATPase transporters hydrolyze the ATP to move the nutrients through the cell membranes (Axelsen and Palmgren, 2001), some P-type ATPase are involved in the transport of metals through the cell membranes. Among these, the P1B ATPase transporters are involved in the transport of heavy metals (Cu, Zn, Co, Cd, Pb) through the cell membranes (Axelsen and Palmgren, 2001; Arguello et al., 2007). 3) In terms of Na^+/H^+ antiporters, the antiporter Na^+/H^+ maintains the cell homeostasis by exporting or importing Na^+ outside or inside the cell (Padan et al., 2001), although the Na^+/H^+ antiporter is not involved in the transport of metals, they are of great importance because they control the cellular homeostasis which is being imbalanced by the uptake of heavy metals.

Thus, the aim of the present study, was to confirm the ability of *S. minima* to accumulate lead in its tissues, and its effect on the transcripts levels of transporter genes, apparently involved in detoxification mechanisms, which may play an important role in protecting this fern against lead stress.

2. Materials and methods

2.1. S. minima culture conditions and lead exposure

Plants of the aquatic fern *S. minima* Baker (Salviniaceae) ecotype Yucatan, were collected from the regional botanic garden Roger Orellana (21°1´N, 89°38´W, Merida, Yucatan, Mexico). The plants were cultivated in hydroponics conditions in a modified Hoagland´s solution (Hoffmann et al., 2004) with pH 6, at 25 ± 2 °C, in a culture room with a photon flux density 100 μmol $m^{-2} s^{-1}$, a relative humidity of 70 ± 5%, an artificial light photoperiod of 12 h. Plants were cultured under those conditions for one week, before they were used for the present experiment. Plants with intermediate development (adult plant), and homogenous characteristics, were divided into 3 different groups. One group was transferred to aqueous solutions containing 0 μM of $Pb(NO_3)_2$, (control plants). A second group was transferred to aqueous solution containing 40 μM of $Pb(NO_3)_2$ (where they remained for 24 h). And a third group where plants were exposed to 40 μM of Pb $(NO_3)_2$ for 24 h, but they were then removed to a lead-free fresh solution (where they remained for further 24 h). In all treatments, the plants remained in a culture room inside plastic vessels (Magenta, SIGMA), each of 6.5 × 6.5 × 10 cm under similar environmental conditions (temperature, light, photoperiod, RH).

2.2. Quantification of Pb from the medium and from plant tissues

The quantification of total Pb in the medium and tissues at each sampling time for all the three treatments were made by atomic emission spectrometer inductively coupled to plasma (ICP-OES, Varian series 730-es; Agilent Technologies Inc. Palo Alto, USA). The detection of Pb was carried out by readings at 220.353 nm using 1% HNO_3 as blank. A standard solution for lead ions (100 ppm) was prepared from analytical grade of $Pb(NO_3)_2$ (Sigma, USA). The working standards were prepared by serial dilution of the standard stock solution for spectrometer calibration 50, 20, 5, 2, and 0.5 ppm of Pb (Hoffmann et al., 2004).

All medium samples (3 mL) were acidified (pH < 2) with 0.2% HNO_3 solution, before being stored at −20 °C for further analysis. 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 (Hoffmann et al., 2004). Plants of *S. minima* from all treatments were separated into leaf-like fronds (leaves) and roots-like fronds (roots). Then, the tissues were stored at −80 °C. Tissues were freeze-dried and 20 mg were mixed and digested with 200 μl HNO_3 (69%) for 3 h at 80 °C in a sand bath inside tightly closed 2 mL Eppendorf reaction tubes (Zauke et al., 1996). The completely digested biomass was brought up to 2 mL with deionized water and vortexed for 10 min. The tubes were centrifuged at 13,200 rpm for 15 min before being stored at −20 °C for further analysis. The Bioconcentration factor (BCF) defined as [Pb concentration (mg kg^{-1} DW) in tissues/Pb concentration initial (mg L^{-1}) in the solution] (Zayed et al., 1998). The Pb translocation factor (TF) defined as [Pb concentration in leaf (mg kg^{-1} DW)/Pb concentration in root (mg.kg^{-1} DW)] (Li et al., 2012). The delta uptake (Δ) defined here as [Pb concentration (mg g^{-1} DW) in tissues at time 2 – Pb concentration (mg g^{-1} DW) in tissues at time 1].

2.3. S. minima ESTs, cDNAs and mRNA sequence

The genes of the transporters involved in accumulation and detoxification system in *S. minima* were identified using in silico approach. The published *S. minima* ESTs genes (NCBI-GenBank database) were used for select and compare the transporters with sequences in DDBJ/EMBL/GenBank database. ABC transporter sequence of C subfamily (MRP genes), ABC transporter sequence of G subfamily, ATPase transporter sequence of P3A family and Antiporter Na^+/H^+ sequence was identified by sequence similarity search using the conserved regions. Out of the sequences that showed similarity by BLASTx (www.ncbi.nlm.nih.gov) seven plants species were selected which were having high similarity for alignment by MEGA5 (Tamura et al., 2011). Identical amino acids in the alignment were shaded with black and conservative substitutions with gray using the BioEdit (Hall, 1999). The phylogenetic tree was constructed by the maximum likelihood method with *SmABCC* (JTT + G), *SmATPase* (cpREV + G), *SmNhaD* (cpREV + I) and *SmABCG* (cpREV + G) by MEGA5 (Tamura et al., 2011). Bootstrap analysis was performed with 1000 replicates to evaluate the reliability of the interior nodes for a particular grouping pattern in the tree. The conserved domains for the all ESTs were identified using the conserved domain search tools by NCBI. The sequences were classified by Gene Ontology (GO) analysis (E value ≤ 10^{-3}) and by Blast2GO (Conesa et al., 2005). The sequences were also analyzed to get annotations according to the KEGG database and reconstruction of KEGG pathways (Kanehisa and