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Review

Characterization of genes expressed in response to cadmium exposure in the earthworm *Eisenia fetida* using DDRT-PCR

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The transition metal cadmium is a pervasive and persistent environmental contaminant that is both a human toxicant and a carcinogen. To inhibit cadmium-induced damage, cells increase the expression of genes encoding stress-response proteins. The transcription of many stress-responsive genes, including those that encode metallothioneins, glutathione-S-transferases (GSTs) and heat shock proteins have been reported. The aim of this work was to investigate in *Eisenia fetida* the genes whose expressions are regulated following exposure to cadmium. mRNA differential display reverse transcription-polymerase chain reaction was used to analyze gene expression in *E. fetida* exposed to 50 mg/l cadmium solution. Among the derived cDNA clones sequenced, we found 15 genes up-regulated and 12 down-regulated in response to cadmium exposure. The translated amino acid sequences of eight clones were similar to the *Lumbricus terrestris* hemoglobin dodecamer, *Tribolium castaneum* membrane protein, *Escherichia coli UMN026* DNA-binding transcriptional activator, *Brugia malayi* immunoglobulin, *Homo sapiens* cell growth-inhibiting protein, *Apis mellifera* poly U binding factor, *Escherichia fergusonii* copper transporter, and the mRNA that encodes *E. coli* K-12 cytoplasmic insertase into membrane protein. Five cDNA fragments presented no homology with known gene sequences, suggesting that these sequences may either encode proteins not yet identified or correspond to untranslated regions of mRNA molecules. In-depth functional analyses of these genes are needed to reveal their exact roles.

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1. Introduction

The application of molecular biology techniques to ecotoxicology has allowed for a better understanding of the mechanisms of action of contaminants in living organisms. Indeed, gene expression profiles represent the first level of integration between environmental stressors and the genome, which, through the synthesis of proteins, pilots the response of the organisms to external changes (Brulle et al., 2008a).

It is essential for biological systems to acquire trace metals for growth, respiration, internal signaling, and a myriad of other indispensable activities. However, the consequence of this deep-rooted dependency is that they must avoid being damaged by excessively high levels of reactive and potentially dangerous essential metals (*e.g.* copper) as well as toxic non-essential metals that enter cells and tissues as analogs of essential elements (Brulle et al., 2008a). Cadmium is a metal used primarily in metal coatings and nickel–cadmium batteries; it is also continuously introduced into the atmosphere and soil through the smelting of ores, the burning of fossil fuels, waste incineration, urban traffic, and as a by-product of phosphate fertilizers (Carginale et al., 2002; Liao and Freedman, 1998; Sanità di Toppi and Gabbrielli, 1999). The existence of a group of genes whose expressions varied following cadmium exposure have been found in *Caenorhabditis elegans* (Liao and Freedman, 1998; Cui et al., 2007). As a key representative of the soil fauna, earthworms are essential in maintaining soil fertility through their burrowing, ingestion, and excretion (Edwards, 2004). Earthworms are increasingly recognized as indicators of agroecosystem health and an ecotoxicological sentinel species because they are constantly exposed to soil contaminants (Pirooznia et al., 2007). Heavy contaminations of soils are a threat to public health. Cases of cancer related to high cadmium concentrations have been described (Nawrot et al., 2006).

In this report, we describe the characterization of gene expressions following cadmium exposure in the ecotoxicologically important earthworm species *Eisenia fetida*. This is only the second animal in which phytochelatin has been identified and induction by heavy metals has been characterized (Brulle et al., 2008b). Recently, the high degree of conservation among defense protein actors has allowed the cloning and study of the expression of 14 effectors in the laboratory model *E. fetida* using real-time polymerase chain reaction (Brulle et al., 2006). This oligochaete is known to express a metal-inducible Class II metallothionein (Brulle et al., 2006; Demuynck et al., 2006). Being ecologically important species, earthworms have been used extensively as test organisms in terrestrial ecotoxicology and more recently in genomics research (Stürzenbaum et al.,1998; Pirooznia et al., 2007; Owen et al., 2008; Svendsen et al., 2008). In the absence of full genome sequences, expressed sequence tags (ESTs) allow for the rapid identification of expressed genes by sequence analysis; they are thus an important resource for comparative and functional genomic studies. To understand the gene expression response to cadmium contaminants, we cloned and sequenced putative differentially expressed fragments from differential display reverse transcription-PCR (DDRT-PCR) and further confirmed their expression patterns using reverse Northern blot analysis.

2. Materials and methods

2.1. Earthworm material and metal application

E. fetida earthworms were obtained from an earthworm farm at China Agriculture University, and kept in the test substrate (cattle manure) in laboratory for a week prior to starting the experiment. The individual earthworms were adults with well-developed clitella. In the laboratory, the earthworms were immersed in double distilled water containing 50 mg/l Cd. All the concentrations are calculated on Cd^{2+} ions. All the solutions were prepared with distilled water and the chloride salt formulation of cadmium ($CdCl_2 \cdot 2.5\ H_2O$; reagent grade). Control earthworms were immersed in ddH_2O. The exposure times were 1, 2, 4, 6, and 8 h.

Cadmium was determined by flame atomic absorption spectrometry (FAAS) (Mulgrave, VIC, Australia, Spectra AA-20). Cadmium in whole worms (3 groups of 5 animals) were determined after acid digestion and 30% v/v hydrogen peroxide. After digestion, the samples were dried on a hot plate (ca. 120 °C) to near dryness, filtered through quantitative filter paper and diluted to 25 ml with deioninzed water.

2.2. RNA isolation and purification

At the end of the cadmium treatment, groups of 20 earthworms were removed quickly, snap frozen in liquid N_2 and stored at −80 °C until use. Total RNA was extracted from the collected whole worm samples using TRIzol® Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. After deoxyribonuclease I treatment, the quantification and purity of RNA samples were assessed using ultraviolet spectrophotometry; the integrity was verified by electrophoresis on 1% agarose gels stained with ethidium bromide. The RNA pellets were stored at −80 °C until needed. Total RNA (20 μg) was treated with 10 units RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) in a 25 μl reaction volume at 37 °C for 30 min. The RNA pellets were washed in 70% ethanol and suspended in 10 μl DEPC-treated ddH_2O just before use.

2.3. First-strand cDNA synthesis

The first strand of cDNA was synthesized following the manufacturer's recommendations for TransScript First-Strand cDNA Synthesis SuperMix (Transgene, SP, China), using 1 μl of μg ml^{-1} oligo(dT) primer AP in a 20 μl reaction. The cDNA was diluted 1:10 and stored at −20 °C for subsequent PCR.

2.4. Differential display RT-PCR

Differential display RT-PCR (Liang and Pardee, 1992) was performed on single-stranded cDNA using combinations of 10 random primers (AP1–AP10) and 3 anchored primers (Table 1). Samples of cDNA from different time points (0, 1, 2, 4, 6, and 8 h after the addition of cadmium) were synthesized appropriately in equal amounts for use in DDRT-PCR experiments. The 25 μl PCR reactions contained 2 μl cDNA template, 1 μl anchor primer, 1 μl arbitrary primer, 12.5 μl 2 × TransTaq PCR SuperMix, and RNAse-free ultrapure pure water (Transgene). Cycling conditions consisted of an initial denaturation for 2.5 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C; followed by a final extension for 5 min at 72 °C. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under ultraviolet light. Differences in band intensities were considered to be caused by differential expression. Intense bands were identified visually (Fig. 2) and selected for cloning.

2.5. Re-amplification

The fragments identified by visual examination as differentially expressed bands were excised from the gels by aligning them to the autoradiograph and suspended in 10 μl ddH_2O. Gene fragments were re-amplified by PCR with the same primer combinations used for the DDRT-PCR, as follows: initial denaturation for 2.5 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C; followed by a final extension for 5 min at 72 °C. The re-amplified products were visualized on 1% agarose gels and the bands recovered using a DNA gel extraction kit (Axygen, Union City, CA, USA).

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