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Highlighted Article

cDNA cloning and expression analysis of *Eisenia fetida* (Annelida: Oligochaeta) phytochelatin synthase under cadmium exposure

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Abstract

Metallothioneins (MTs) are central to trace metal homeostasis and detoxification throughout biological systems. Prokaryotes, plants, and fungi utilize both gene encoded cysteine-rich polypeptides (classically designated Class I and II MTs) and enzymatically synthesized cysteine-rich peptides (classically designated Class III MTs or phytochelatins). In contrast, although gene encoded MTs are ubiquitous in animal species the identification of a functional phytochelatin synthase in the nematode *Caenorhabditis elegans*, a representative member of the Ecdysozoa, provided the first evidence for these metal-binding peptides in animals. By exploiting the conservation observed between species we have been able to clone and transcriptionally characterize a phytochelatin synthase from the immune cells of the earthworm *Eisenia fetida*, the first evidence for its presence in a phylum belonging to the Lophototrochozoa. The complete coding sequence of this enzyme was determined and the phylogenetic relationship to plant, yeast and nematode enzymes elucidated. Temporal- and dose-profiling of the transcriptional regulation of phytochelatin synthase and MT in response to cadmium was performed by using real-time PCR.

Keywords: *Eisenia fetida*; Phytochelatin synthase; Cœlomocytes; Cadmium exposure; Expression; Real-time PCR

1. Introduction

It is essential for biological systems to acquire essential trace metals for growth, respiration, internal signalling, and a myriad of other indispensable activities. However, the consequence of this deep-rooted dependency is that they must avoid being damaged either by excessively high levels of reactive and potentially dangerous essential metals, like copper, as well as toxic non-essential metals that enter cells and tissues as analogues of essential elements. Metallothioneins (MTs) are a ubiquitous family of cysteine-rich proteins/peptides that chelate metal ions providing protection against metal cytotoxicity whilst ensuring the trafficking of metals to their intended targets (Vasak and Hasler, 2000). The functional linkage between MTs and metal detoxification has facilitated their exploitation as diagnostic markers for metal contamination (Kim et al., 2006). Classically MTs were divided into three classes, the gene which exhibit high or no homology with horse MT, Class I and II MT, respectively, and those enzymically synthesized, Class III MTs or phytochelatins (Fowler et al., 1987). This classification system has been refined to uncouple the phytochelatins from the exponentially expanding phylogenetically diverse family of gene encoded MTs (Cobbett and Goldsbrough, 2002).

In plants, metal tolerance has classically been associated with the synthesis of the post-transcriptionally synthesized phytochelatins (poly-[γ-Glu-Cys]$_n$-X polymers) (PCs) (Grill

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et al., 1985; Cobbett, 1999; Cobbett and Goldsbrough, 2002). Indeed, since their discovery in the yeast *Schizosaccharomyces pombe* in 1985, PCs have been characterized in a wide range of plant species (Grill et al., 1985) and play a major role in the detoxification of trace metals in plants by chelating metals, especially cadmium, with high affinity. PCs have also been implicated in the regulation of intracellular essential metal concentrations in plants, fungi, and microalgae (see Clemens, 2006). In higher plants, these peptides are synthesized after exposure to certain metal trace elements and form macro-molecular complexes facilitating metal detoxification (Hirata et al., 2005). PCs are synthesized by phytochelatin synthase (PCS), or γ-Glutamyl cysteine dipeptidyl transferase, an enzyme which catalyses the transfer of a γ-Glu-Cys unit of a glutathione molecule either to another [γ-Glu-Cys-Gly + γ-Glu-Cys-Gly → (γ-Glu-Cys)$_2$-Gly + Gly] or to a molecule of phytochelatin previously synthesized to generate a polymer containing from 2 to 11 repeated γ-Glu-Cys units (Grill et al., 1989). A study performed on garlic (*Allium sativum*) in 2005 using semi-quantitative RT-PCR showed that phytochelatin synthase was controlled at the transcriptional level following exposure to metals, especially cadmium (Zhang et al., 2005). Indeed, these authors showed that *pcs*-gene expression in the roots of *Allium sativum* was significantly induced by cadmium-induced stress.

In most animals, tolerance to heavy metals depends on the induction of genomically encoded MTs, a family of cysteine-rich proteins of low molecular weight (6000–8000 Da) characterized by the absence of aromatic amino acids and limited homology to horse MT (Fowler et al., 1987). These metallo-proteins are known to be implicated in the detoxification process of heavy metals such as cadmium (Cd) and in the homeostasis of essential metals such as zinc (Palmiter, 1998; Klaassen et al., 1999). Estimating the impact of metals on soil macroinvertebrates by quantifying transcript levels of genomically encoded Class II MTs has been performed in a number of terrestrial species, including the oligochaete worms *Lumbricus rubellus* (Stürzenbaum et al., 2004) and *Eisenia fetida* (Demuynck et al., 2005; Brulle et al., 2006; Demuynck et al., 2006), the nematode *Caenorhabditis elegans* (Liao and Freedman, 1998), and the springtail *Orchesella cincta* (Timmermans et al., 2005).

Prior to 2001 there had been no reports of the presence of PCs or phytochelatin synthase in any animal. However, soon after the completion of the full genome sequence for the nematode *C. elegans* two publications independently described a functional PCS in this model invertebrate (Clemens et al., 2001; Vatamaniuk et al., 2001). Moreover, Clemens et al. (2001) hypothesized that PCs may be ubiquitously involved in the tolerance and homeostasis of metals in all eukaryotic organisms, a hypothesis which is yet to be tested.

In this paper we describe the cloning and the cadmium-dependent transcriptional control of a phytochelatin synthase from the ecotoxicologically important earthworm species *E. fetida*. This is only the second animal in which *pcs* has been identified and induction by heavy metals characterized. This oligochaete has been exploited by the OECD since 1984 for the ecotoxicological assessment of chemicals (OECD, 1984; Dominguez, 2004), and it is known to express a metal-inducible Class II MT (Demuynck et al., 2005; Brulle et al., 2006; Demuynck et al., 2006). We have investigated the complementary transcriptional induction of *mt* and *pcs* genes in response to cadmium within *E. fetida* and discussed their possible interdependency and potential implications for metal detoxification.

2. Material and methods

2.1. Animals and treatment

Earthworms came from controlled cultures maintained in our laboratory. Worms were bred at an ambient temperature of 22 ± 2 °C, in the dark, on vegetable mould with fresh cattle manure as food source. The moisture content was maintained around 60%.

Artificial substrates were prepared as described in OECD guideline (OECD, 1984). Briefly, substrate was composed (percentages refer to dry weight) of 10% sphagnum, 20% kaolinite clay, and 70% quartz sand. The pH was adjusted to 6 ± 0.5 with $CaCO_3$. The cadmium ($CdCl_2$) was added to the soil. Then, half an hour later, worms were added.

Three concentrations (8, 80, and 800 mg/kg) of cadmium were tested and 25 adult worms per condition were used. The latter concentration, although very high is under the LC50 (1000 mg/kg) reported for *E. fetida* in OECD artificial soil (Honeycutt et al., 1995). Exposure periods were 2 and 14 days. At the end of exposure periods, worms were rinsed in distilled water before coelomocyte extrusion, with non-invasive method using cold extrusion medium (2.5 mg ml^{-1} EDTA, 8.5 mg ml^{-1} NaCl, pH 7.3, 5% ethanol) and electrical stimuli as previously described (Brulle et al., 2006). Then cellular RNA extraction was processed as described below.

2.2. RNA extraction and reverse transcription

All expression analyses were conducted on total RNA extracted from cœlomocytes, using Tri-Reagent® (Molecular Research Center, Inc., Cincinnati, USA) according to manufacturer's instructions. RNA purity and integrity were checked by ensuring that absorbance ratios (A260/280) were between 1.8 and 2.0 and by agarose gel electrophoresis (2%). First strand cDNA synthesis was performed using 200 units of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas, Burlington, Canada), 200 ng of random primer, and 1.5 μg of total RNA in order to allow the cloning using degenerate primers. For real-time PCR analysis, reverse transcription was conducted with 200 units of SuperScript II reverse transcriptase™ (GibcoBRL, Cergy-Pontoise, France), 500 ng oligo (dT) primer (18-mer) and 1.5 μg total RNA. The use of oligo (dT) priming within the reverse transcription bias cDNA synthesis towards mRNA and consequently the protein encoding RNA population.

2.3. Cloning and sequencing

Cloning of the *pcs* gene was achieved using degenerate primers designed against evolutionarily conserved motifs of the enzyme. To identify these conserved sequences, protein sequences of PCS (already cloned in other eukaryotic species, mainly plants and *C. elegans*) were obtained from GenBank (NCBI). Alignments were done with the online/Web tools (MULTALIN; CLUSTAL W) (Corpet, 1988; Thompson et al., 1994). Primers were then determined using software available online (CODEHOP) (Rose et al., 1998). The forward (5′-CCAGAACGAGCCCG-C(AGCT)T(AT)(CT)TG(CT)GG-3′) and reverse (5′-GTGGGGAGGG-TACTTGAACC(GT)(AGCT)GC(AGCT)AC(AG)TC-3′) primers were designed to minimize their level of degeneracy and optimize their