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Effect of cadmium on gene expression in the liverwort *Lunularia cruciata*

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Abstract

Bryophytes are valuable bioaccumulators, because they have high absorbing and ion exchange capacities. Cadmium (Cd) is a heavy metal naturally present in soil; it is non-essential and highly toxic to most organisms, having toxicity 2 to 20 times higher than many other heavy metals. The presence of elevated levels of Cd ions triggers a wide range of cellular responses including changes in gene expression and synthesis of metal-detoxifying peptides. To investigate the ability of Cd to affect gene transcription, the messenger RNA (mRNA) differential display technique was applied to the identification and isolation of genes whose transcription was altered in cultured *Lunularia cruciata* plants that were grown in the presence of cadmium salts. Four genes whose mRNA levels significantly changed in response to cadmium exposure were isolated and identified. The first gene identified in our analysis is up-regulated by Cd: it encodes the enzyme cystathionine γ-synthase. The other genes are down-regulated by cadmium. These genes encode a methyltransferase, a tyrosine phosphatase and the EST 408 of the diatom *Fragilariopsis cylindrus*, whose function is unknown. Our findings demonstrate the usefulness of mRNA differential display technique for the detection of plant metabolic pathways affected by cadmium stress.

Keywords: Differential display; Stress response; Protein phosphorylation; Cysteine biosynthesis; Methyltransferase; Bryophytes

1. Introduction

Heavy metal ions play essential roles in many physiological processes. In trace amounts, several of these ions are required for metabolism, growth, and development. However, problems arise when cells are confronted with an excess of these vital ions or with nonnutritional ions that are potentially highly toxic to all organisms including animals and plants (Cinquetti et al., 2003).

Numerous studies on the physiological responses to excess amounts of heavy metal ions indicate that plants have developed various mechanisms to cope with this environmental threat. Until now, however, the cellular mechanisms of heavy metal stress-induced signalling remained unclear. Metal ions can affect all the different classes of biomolecules included within the context of the genome, transcriptome, proteome, and metabolome. Here, we have focused our attention to the effects of cadmium on the transcriptome and we have looked for genes whose expression may be influenced by metal exposure. In fact, it is widely recognized that metal compounds may have a profound effect on gene expression patterns, as demonstrated by the growing number of metal responsive genes

Abbreviations: bp, base pairs; cDNA, DNA complementary to RNA; dCTP, deoxycytidine triphosphate; DDRT-PCR, differential display reverse transcription polymerase chain reaction; DNase, deoxyribonuclease; dNTP, deoxyribonucleoside triphosphate; EMBL, European Molecular Biology Laboratory; MMLV, Moloney murine leukemia virus; oligo(dT), oligodeoxyribonucleotide thymidine; RNase, ribonuclease; RT, reverse transcriptase; SDS, sodium dodecyl sulphate; SSC, 0.15 M NaCl/0.015 M Na_3.citrate pH 7.6; UV, ultraviolet.

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that have been identified in different organisms (Hall, 2002; Jonak et al., 2004).

Cadmium (Cd) is a widespread heavy metal; it is non-essential and highly toxic to most organisms, having toxicity 2 to 20 times higher than many other heavy metals (Vassilev et al., 1998). It has a long biological half-life and accumulates in liver and kidney mostly under the form of a complex with metallothionein. The carcinogenic potential of cadmium is recognized beyond any doubt both in experimental animals and in humans; however, the mechanism of cadmium carcinogenesis is still debated and basically unsettled (Beyersmann, 2002). In plants, Cd causes leaf roll and chlorosis, interacts with water balance and damages the photosynthetic apparatus (Siedlecka and Krupa, 1996). Cd also significantly reduces the normal H^+/K^+ exchange and the activity of plasma membrane ATPase (Obata et al., 1996), and strongly affects the activity of several enzymes (Mattioni et al., 1997).

Bryophytes have a high capacity to accumulate heavy metals because of the high surface/volume ratio, absence or reduction of a cuticle, high absorbing and ion exchange capacity on their cell wall, and simple anatomic organization (Salemaa et al., 2004). They have no root system and their leaves are only a one cell-thick-layer, thus they get the nutrients and elements as well as heavy metals mainly from wet and dry depositions (Vargha et al., 2002). Hence, mosses are widely used as bioindicators of atmospheric fallout (Carballeira et al., 2001) and as models for morphological and genomic alteration caused by heavy metals (Carginale et al., 2004a). In recent years, bryophytes have also been increasingly used to monitor pollution in aquatic environments, such as streams and lakes, and are currently studied in relation to uptake as well as extracellular or intracellular compartmentalisation of heavy metals (Vazquez et al., 1999; Nimis et al., 2002).

Differential display (DDRT-PCR) is a powerful technique used to rapidly identify and isolate genes that are differentially expressed between two cellular populations, or within a single cell type under altered conditions (Liang and Pardee, 1992; Carginale et al., 2004b). In this paper, we have applied the differential display technique to explore how exposure to cadmium might affect gene expression in the liverwort *Lunularia cruciata*.

2. Materials and methods

2.1. Gametophyte cultures

Field-grown *L. cruciata* (L.) Dum. was gathered in the Botanical Garden of University Federico II, Naples. Single gametophytes were thoroughly washed with deionised water and cultured in Petri dishes (5-cm diameter), 20 specimens per dish, on discs of paper wetted with 2 ml of sterile distilled water (control) or 10^{-4} M $CdCl_2$ for 2 weeks. The cultures were kept in a climatic room with a temperature ranging from 13 to 20 °C (night and day temperature within the chamber), 70% constant relative humidity, and a photoperiod of 16-h light (40 mEinstein/m^2/s) and 8-h dark.

2.2. Extraction of total RNA

At the end of the Cd treatment, plants were washed in deionised water, immediately frozen in liquid N_2 and grinded to a fine powder in a prechilled mortar with a pestle. Total RNA was then isolated from 0.1 g nitrogen-dried powder of control and Cd-treated samples of *L. cruciata* using the GenElute Total RNA kit (Sigma). After removal of contaminating genomic DNA using the DNA-free kit (Ambion), the concentration and purity of RNA samples were determined by UV absorbance spectrophotometry.

2.3. cDNA synthesis and differential display RT-PCR

Differential display (Liang and Pardee, 1992) was done using the Delta Differential Display Kit (Clontech Laboratories, Inc.) following the manufacturer's instructions. DNA-free total RNA (4 μg) extracted from pooled plants of control and Cd-treated *L. cruciata* was reverse-transcribed in 10 μl of RT buffer (50 mM Tris–HCl, pH 8.3, 6 mM MgCl2, 75 mM KCl), containing 1 mM of each dNTP, 0.1 μM cDNA synthesis primer (oligo-dT) and 200 units of MMLV-reverse transcriptase. The samples were incubated at 42 °C for 60 min, then heated at 75 °C for 10 min to inactivate the reverse transcriptase. Each first-strand cDNA sample obtained was diluted 1:4 (dilution A) and 1:16 (dilution B) and stored at −20 °C for subsequent PCR reactions. Amplification of cDNA fragments was performed in 20 μl PCR reactions, each in the presence of one of the 90 possible combinations of arbitrary upstream (designed as "P") and downstream (designed as "T") primers supplied by the manufacturer. The sequences of P and T primers used in these reactions are reported in Table 1. Each reaction mixture contained 1 μl first-strand cDNA, 1X PCR buffer [50 mM Tris–HCl, pH 8.3, 10 mM KCl, 5 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$], 50 μM dNTPs, 2 μCi [α-^{33}P]dATP (Amersham Pharmacia Biotech), 1 μM of P primer, 1 μM of T primer and 1 unit of FastStart Taq DNA polymerase (Roche). Reactions were carried out in a DNA Thermocycler Express (Hybaid) using the following program: 1 cycle at 94 °C for 4 min, 40 °C for 5 min, 72 °C for 5 min; 2 cycles at 94 °C for 1 min, 40 °C for 1 min, 72 °C for 5 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min. This was followed by a final elongation step at 72 °C for 7 min. The PCR products obtained from dilution A and B of each first-strand cDNA produced from RNA of control and Cd-treated plants were separated in parallel by denaturing electrophoresis in 6% polyacrylamide/8 M urea gels. After electrophoresis, gels were dried onto Whatman 3MM paper and exposed to Fuji X-ray film