Mol Biol Rep (2013) 40:2157–2167
DOI 10.1007/s11033-012-2275-7

Effects of cadmium exposure on sea urchin development assessed by SSH and RT-qPCR: metallothionein genes and their differential induction

Maria Antonietta Ragusa · Salvatore Costa · Marco Gianguzza · Maria Carmela Roccheri · Fabrizio Gianguzza

Received: 3 August 2012 / Accepted: 19 November 2012 / Published online: 2 December 2012

Abstract In order to study the defense strategies activated by *Paracentrotus lividus* embryos in response to sublethal doses of $CdCl_2$, we compared the induced transcripts to that of control embryos by suppression subtractive hybridization technique. We isolated five metallothionein (MT) cDNAs and other genes related to detoxification, to signaling pathway components, to oxidative, reductive and conjugative biotransformation, to RNA maturation and protein synthesis. RT-qPCR analysis revealed that two of the five *P. lividus* MT (*Pl*MT7 and *Pl*MT8) genes appeared to be constitutively expressed and upregulated following cadmium treatment, whereas the other three genes (*Pl*MT4, *Pl*MT5, *Pl*MT6) are specifically switched-on in response to cadmium treatment. Moreover, we found that this transcriptional induction is concentration dependent and that the cadmium concentration threshold for the gene activation is distinct for every gene. RT-qPCR experiments showed in fact that, among induced genes, *Pl*MT5 gene is activated at a very low cadmium concentration (0.1 μM) whereas *Pl*MT4 and *Pl*MT6 are activated at intermediate doses (1–10 μM). Differently, *Pl*MT7 and *Pl*MT8 genes increase significantly their expression only in embryos treated with the highest dose (100 μM $CdCl_2$). We found also that, in response to a lethal dose of cadmium (1 μM), only *Pl*MT5 and *Pl*MT6 mRNA levels increased further. These data suggest a hierarchical and orchestrated response of the *P. lividus* embryo to overcome differential environmental stressors that could interfere with a normal development.

Keywords Cadmium · Echinodermata · Gene expression · Metallothionein · Multigene families · Embryonic development

Introduction

Humans, animals and plants are exposed to a variety of metals dispersed in the environment and contained in water and food. Heavy metals are non essential non-biodegradable chemical elements that persist in the environment for long periods, causing serious eco-toxicological problems. In addition, some of these toxic metals may mimic essential metals.

Cadmium (Cd) is a heavy metal that is toxic for all living organisms even at low concentrations, whose presence in the environment has grown, in time, because of its large employment in some industrial and agricultural activities.

Cd does not have any biological role but, since it cannot be degraded, it is irreversibly accumulated into cells, interacting with cellular components and some molecular targets.

The toxic effect of Cd has been associated in general with blockage of oxidative phosphorylation, glutathione depletion and antioxidant enzymatic activity inhibition,

Electronic supplementary material The online version of this article (doi:10.1007/s11033-012-2275-7) contains supplementary material, which is available to authorized users.

M. A. Ragusa (✉) · S. Costa · M. Gianguzza · M. C. Roccheri · F. Gianguzza
Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari, Università di Palermo, viale delle Scienze, Ed. 16, 90128 Palermo, Italy
e-mail: maria.ragusa@unipa.it

F. Gianguzza
e-mail: fabrizio.gianguzza@unipa.it

production of ROS, DNA damage, and inhibition of relative repair mechanisms. It is also well known that all invertebrates and vertebrates, when exposed to heavy metals, usually activate protection systems by increasing the expression of both metal binding proteins such as metallothioneins and heat shock proteins.

Although heavy metals are terrestrially produced, they flow into the sea through effluents and sewage or are directly discharged from industries placed on the seawater front.

Aquatic invertebrates can to accumulate, in non toxic forms, high levels of heavy metals in their tissues and yet survive in polluted environments [1, 2].

The toxic effects of high Cd concentration have been studied in embryos of marine invertebrates. Sub-lethal effects of lower Cd doses on population growth rate, gametogenesis and embryogenesis have been also described in various species of aquatic invertebrates (for review, see [3]).

Among the benthonic organisms, echinoderms represent a simple, but significant, model system to test how a specific stress can singly and/or simultaneously affect development and putative mechanisms of defense and/or cell death [4–8].

The position of echinoderms in the marine trophic chain, where pelagic larvae are part of the diet of several planktonic and benthonic organisms, has furthermore increased the interest of many researchers. In addition, because the gametes, embryos, and larvae of sea urchin are sensitive to various heavy metals, they are usually used in toxicological studies as environmental indicators; both as short-term indicators (due to the short duration of its larval life stages), and also as long-term indicators (during their sedentary post-metamorphic life).

Finally, considering the capacity of sea urchin embryos and larvae to accumulate contaminants during development, this developmental model offers an excellent opportunity to investigate the possible adaptive response of cells exposed to Cd during differentiation and during all developmental stages and/or life cycle [9–11].

In particular, one of us has previously shown that *Paracentrotus lividus* sea urchin embryos exposed to 1 mM $CdCl_2$ undergo to development malformation, development arrest and/or cell death [9, 10, 12, 13], whereas, if exposed to lower Cd concentrations (i.e. 100 μM $CdCl_2$), *P. lividus* embryos are able to activate a variety of cellular sensory and detoxification pathways that monitor and counteract potentially toxic heavy metals and that can allow embryo survival, such for instance heat shock protein (HSP) synthesis, Metallothionein (MT) synthesis and accumulation, and autophagy/apoptosis induction [8–10, 14–18].

The study reported in this paper is aimed to clarify the early defense strategies activated by *P. lividus* 30 h embryos, in response to sub-lethal doses of Cd (100 μM $CdCl_2$) [8–11, 15, 16], analyzing the induced transcripts and comparing it to that of control (untreated) embryos by suppression subtractive hybridization (SSH) technique.

Table 1 PCR primers and target genes

Target	Oligo sequence (5′– 3′)	Oligo name
Adapter primer	TCGAGCGGCCGCCCGGGCAGGT	PCRPRIM1
	AGCGTGGTCGCGGCCGAGGT	PCRPRIM2R
*Pl*Talpha1 coding region	CTTATCTTCCACAGCTTCGGT	Alpha-Tub For
	GGTGGGACGGTCGATGTC	Alpha-Tub Rev
*Pl*Talpha1 5′ UTR and 3′ UTR regions	GAAGACACCTGGGATATCACC	Alpha1 5′UTR For
	GCAGTCCTGTGGTTTCTATC	Alpha1 3′UTR Rev
Metallothionein 4	GCTCAAAATCTTCAACATGGCTAATGA	MT4 For
	AGCACTTTCCAGTTTCACAACAAGC	MT4 Rev
Metallothionein 5	CGACTTTAGCTCAAATTCATCACCATG	MT5 For
	TCCACAGCATTTACCATCCTTGC	MT5 Rev
Metallothionein 6	CACGATTTGTGCTCAATCCTTCAT	MT6 For
	TTTGTGCATGATGTTCCACAGC	MT6 Rev
Metallothionein 7	CGTCAAGAGATCAAAATCATCAACCA	MT7 For
	ACAGCACTCGCCAGTAATACAGCAC	MT7 Rev
Metallothionein 8	GATGGTTGTCGTCGCTCCTAACA	MT8 For
	TCAAGAAAGGCTGGTATCAAATCTGAC	MT8 Rev
18S ribosomal RNA	GAATGTCTGCCCTATCAACTTTCG	18S rRNA For
	TTGGATGTGGTAGCCGTTTCTC	18S rRNA Rev

Materials and methods

Embryo cultures and morphological analysis

Gametes were collected from gonads of the sea urchin *P. lividus*, harvested from the West Coast of Sicily. Eggs were fertilized and embryos reared at 18 °C in millipore-filtered seawater in the presence of antibiotics (50 mg/l of streptomycin sulfate and 30 mg/l of penicillin) at the dilution of 5,000/ml in glass beakers. In cadmium-exposure experiments, embryos were continuously cultured after fertilization in the presence of different $CdCl_2$ concentrations (0.1, 1, 10, 100 μM and 1 mM), and their development was monitored up to the stages of interest.

For total ribonucleic acid (RNA) extraction, embryos were collected by low-speed centrifugation at 4 °C and processed as reported in the following section. Morphological analysis of embryo development was performed after immobilization of embryos with 0.1 % formaldehyde (final concentration) in seawater and observation under an Olympus microscope (OSP-MBI).

Total RNA extraction and poly(A)+ purification

Total RNA was extracted from control and $CdCl_2$-treated *P. lividus* embryos at the gastrula stage (30 h for embryos treated with 100 μM $CdCl_2$; or 24 h for embryos treated with 1 mM $CdCl_2$) with the RNeasy Maxi Kit (Qiagen) following the manufacturer's instructions and DNAseI treatment.

The RNA quality was assessed by denaturing agarose gel electrophoresis, and the RNA concentration and purity were determined by measuring the absorbance at 260, 280 and 230 nm (using an Eppendorf biophotometer, data not shown).

Poly(A)+ RNA was prepared by using the Dynabeads® mRNA Purification Kit (Invitrogen) according to the manufacturer's instructions.

mRNA quality was assessed performing RT-PCR reactions (Superscript VILO cDNA synthesis kit, Invitrogen) using alpha1 tubulin 5′UTR and 3′UTR specific primers (Table 1), obtaining the expected 1.5 kb product [20].

Suppression subtractive hybridization

Suppression subtractive hybridization was performed with the PCR Select cDNA Subtraction Kit (Clontech Laboratories, USA) according to the manufacturer's instructions. This strategy is built on a PCR-based method for the selective amplification of differentially expressed sequences, thereby allowing the isolation of some over- and under-expressed transcripts in Cd-treated embryos.

Subtracted forward (Cd-treated—controls: CD—Ctrl) and reverse (Ctrl—CD) reactions were generated by subtracting Ctrl cDNAs from CD cDNAs and CD cDNAs from Ctrl cDNAs, respectively, using the PCR-select cDNA Subtraction Kit (user manual PT1117-1, Clontech Laboratories, Inc., Mountain View, CA, USA). To perform the subtraction reactions, CD and Ctrl cDNAs were digested with RsaI to obtain shorter, blunt-ended molecules suitable for adaptor ligation and optimal for subtractive hybridization.

Subtraction efficiency was evaluated by comparing the abundance of a known gene in subtracted and unsubtracted cDNAs population after different cycles of PCR using EuroTaq DNA Polymerase (Euroclone). *P. lividus* specific primers were designed for a constitutively expressed and not stress-regulated, alpha tubulin gene (*Pl*Talpha1) [19–22]; target cDNA did not contain RsaI restriction site in the amplified sequence. The subtraction efficiency was indicated by the difference in the number of cycles needed to generate equal amplification of the corresponding PCR product before and after subtraction for this gene.

Differentially expressed cDNAs were purified with Nucleon extraction and purification PCR/oligo (Amersham Life Sciences), and cloned in the pGEM-T Easy Vector (Promega). Library screening was performed by colony PCR using adapter specific primers (PCRPRIM1 and PCR-PRIM2R, Table 1). DNA was extracted from selected clones and sequenced with T7 universal primer (Macrogen, inc.).

3′ RACE

For first-strand cDNA synthesis, 2 μg of total RNA, previously extracted from 100 μM $CdCl_2$ treated 30 h embryos, was reverse transcribed using the SuperScript III Reverse Transcriptase and oligo dT (Invitrogen). The PCR was performed with MT4 For (Table 1) and oligo dT primers. The PCR amplification was performed in a 50 μl volume with 1 μl of cDNA, 1× PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTPs, 0.4 μM of each primer, and 0.025 U/μl of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA). The thermal cycling conditions for PCR were 95 °C for 5 min; 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 2 min each; and final extension 72 °C for 5 min.

The PCR product was extracted from 1.5 % agarose gel with the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), cloned in the pGEM-T Easy Vector (Promega) and then sequenced.

Sequences and phylogenetic analysis

The sequences obtained were compared with known sequences using BLASTN software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the nucleotide database: "All GenBank, EMBL, DDBJ and PDB sequences (but not EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS

sequences)". Moreover, using BLASTX software we looked for similar sequences in the protein database: "All non-redundant GenBank CDS translations, PDB, Swiss-Prot, PIR, and PRF excluding environmental samples from WGS projects".

Unidentified short sequences were clustered (when possible) with Codon Code Aligner and were compared with *P. lividus* EST database. Resulting longer sequences were run again in BLASTN or BLASTX. Their putative identity, nucleotide similarity with other known sequences, and insert size are shown in Online Resource 2.

Metallothionein nucleotide coding sequences and protein sequences were aligned with ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/) and multiple sequence alignments were used to construct phylogenetic trees by the neighbor-joining method with PHYLIP software (http://bioweb2.pasteur.fr/docs/phylip/doc/main.html, outgroup was *Tetrahymena pyriformis* MT-2: ABF61447.1), performing 1,000 bootstrap replicates. Phylogenetic trees were visualized with Archaeopteryx (http://www.phylosoft.org/archaeopteryx/). MT amino acid sequences used for Echinodermata MT alignments were derived mostly from amino acid sequence files or translated cDNA open reading frame sequences published in GenBank (http://www.ncbi.nlm.nih.gov/Tools/, see accession numbers in the legend to Figs. 2 and 3.

Reverse transcription and quantitative PCR analysis

First-strand cDNA was synthesized from total RNA (500 ng) by reverse transcription using QuantiTect Reverse Transcription kit (Qiagen). RNAs were extracted from 30 h embryos treated with 0.1, 1, 10, 100 μM $CdCl_2$ or untreated embryos and from 24 h embryos treated or untreated with 1 mM $CdCl_2$. Real-time PCRs were performed in 96 well plates in a 20 μl mixture containing 1 μl of the cDNA preparation diluted fifty times, 10 μl QuantiFast SYBR Green PCR mix (Qiagen), and 1 μM of each primer, in the BIO-RAD CFX96 system using the following PCR parameters: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s. The sequences of the specific primer pairs used for qPCR are shown in Table 1. Specificity of the MT primers was confirmed by cross-PCRs: every MT cDNA was used as template in five different PCR reactions, each one performed using one of the five different MT specific primer pairs (Table 1). All of the subtype-specific primers amplified only the corresponding MT subtype template with the PCR products matching the size of the predicted values (data not shown).

Samples were run in triplicate, with negative controls (no cDNA) and positive controls (100 pg plasmid clone DNA). The absence of nonspecific products was confirmed by both the analysis of the melt curves and electrophoresis in 2 % agarose gels. Amplification efficiency of each primer pair was evaluated and any efficiency correction was loaded in the software.

To obtain sample quantification, the $E^{-\Delta\Delta Ct}$ method was used, and the relative changes in gene expression were analyzed as described in the CFX Manager software manual.

18S rRNA was used as internal reference, in order to compensate for variations in input RNA amounts. Moreover, serial dilution of cDNA (the standard curve method) permitted to calculate target cDNA starting quantity referred to 18S rRNA quantity in arbitrary units (considering 1AU as 18S rRNA/1,000).

Results

Suppression subtractive hybridization

In order to study the early defense strategies activated by *Paracentrotus lividus* in response to sub-lethal doses of Cd, we cultured embryos for 30 h, maintaining them continuously in presence of 100 μM $CdCl_2$, and we carried out a parallel culture of control embryos (same batch of embryos cultured in the same sea water without adding Cd). Embryo morphology was assessed by microscopy: Cd-treated embryos appeared similar to that observed in earlier studies [8, 12]. mRNA was extracted from treated and control embryos and its quality was assessed performing RT-PCR reactions using *Pl*Talpha1 tubulin 5′ and 3′ UTR specific primers (Table 1) [20]. These high quality mRNAs were used for SSH. Subtraction efficiency was evaluated performing semiquantitative PCR on subtracted and unsubtracted cDNA using coding primers (Table 1) for *Pl*Talpha1 tubulin, a constitutively active gene [19, 20] that does not show stress-dependent changes in expression ([21, 22] and personal communication). PCR products were detectable after 25 cycles in the CD unsubtracted sample, whereas PCR fragment was not detected in the CD-Ctrl subtracted sample even after 35 cycles (data not shown). Conversely, the *Pl*Talpha1 tubulin PCR products were detected after 20 cycles in the unsubtracted Ctrl sample and after 15 more cycles in the reverse subtracted Ctrl-CD sample.

Using pGEM-T easy vector, two subtracted cDNA libraries (one forward: CD—Ctrl, and one reverse: Ctrl—CD) were obtained. We isolated a total of about 800 subtracted clones from the forward library and 200 from the reverse library. cDNA clones were screened by colony PCR and clones with insert size >200 bp were selected and sequenced. All 91 cDNA clones (with this feature) were sequenced, 48 were in the 200–400 bp size range, 35 % in the 401–700 bp size range, 4 were >701 bp size (Online Resource 2).

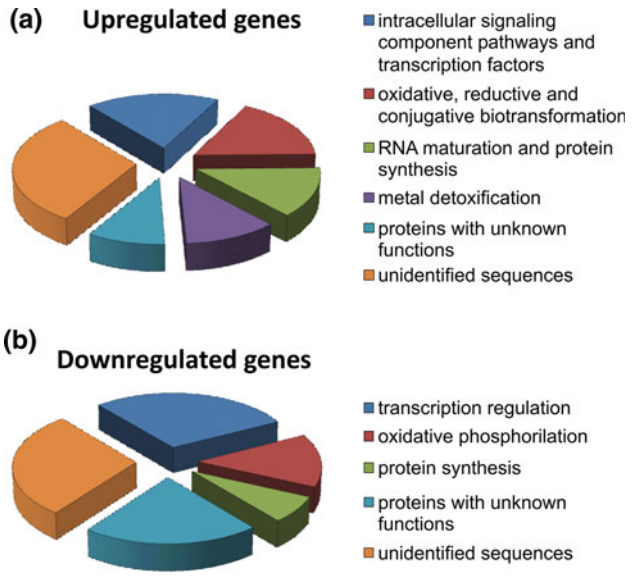

Fig. 1 Functional classification of the expressed genes in each of the two SSH library. **a** up-regulated genes (CD clones); **b** down-regulated genes (Cntrl clones)

The sequences obtained were compared with known sequences in the GenBank (National Center for Biotechnology Information, Bethesda, MD) database (using BLASTN software) or Protein database (using BLASTX software). The sequences were categorized based on their biological pathways (Fig. 1).

In the Cd upregulated group, there were 10 genes related to intracellular signaling component pathways and transcription factors, 9 to oxidative, reductive and conjugative biotransformation, 7 to RNA maturation and protein synthesis, 6 to metal detoxification (including 5 metallothioneins), 5 conserved hypothetical proteins with unknown functions and 16 unidentified sequences (no significant matches).

In the Cd downregulated group, there were 4 genes related to transcription regulation, 2 to oxidative phosphorylation, 1 to protein synthesis, 3 other proteins and 4 unidentified sequences (no significant matches)—(See Online Resource 3).

Metallothionein sequence analysis

Since the main achievement was that 25 % of detected clones codified for metallothioneins (see Online Resource 2), *Pl*MT cDNA sequences were bioinformatically analyzed and grouped in at least five isotypic classes. We named this clones *Pl*MT4 to *Pl*MT8, because three *Pl*MT sequences (here named *Pl*MT1–3) were already in the databases. Moreover, as *Pl*MT4 coding sequence was incomplete, we performed 3′ RACE technique to obtain the full length sequence.

Nucleotide sequences were compared with the sole *Pl*MT mRNA already described [12], here referred to as *Pl*MT1 (Accession number: AJ310190). This analysis showed that *Pl*MT8 could correspond to *Pl*MT1. In fact, the nucleotide sequence alignment between *Pl*MT8 and *Pl*MT1 (performed with BLAST) showed 94 % identity both in ORF and in 3′UTR. Differences are in fact localized principally in 5′ and 3′ end sequences that corresponds to the primers utilized for the previous cloning (Online Resource 1).

Similarly, aminoacid sequence comparison showed only two substitutions: (*Pl*MT8 → *Pl*MT1) at position 5 (Arg → Lys) and position 58 (Val → Gln). Moreover, for sequence comparison the other two protein sequences already published were taken into account [23]. One of these sequences (here named *Pl*MT2) contains Val at position 58, the second

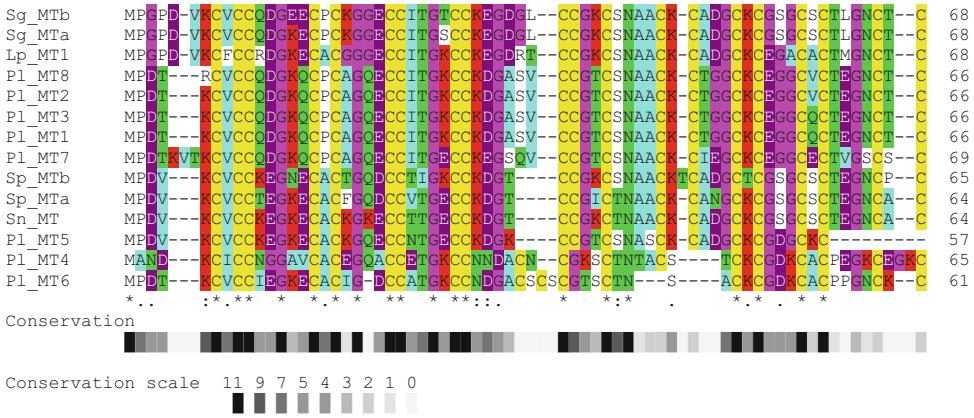

Fig. 2 Protein sequence alignment of Echinodermata metallothioneins performed with ClustalW2. Abbreviation and protein sequences GenBank ID: Sg_MTb: *Sphaerechinus granularis* MT (CAA91784.1), Sg_MTa: *Sphaerechinus granularis* MT (CAA91437.1), Lp_MT1: *Lytechinus pictus* MT (AAB58321.1), Lp_MT2: *Lytechinus pictus* (AAB58320.1) (not aligned: 8 aa only), Pl_MT1: *Paracentrotus lividus* MT (CAC37693.1), Pl_MT2: *Paracentrotus lividus* MT_PARLI (P80367.2), Pl_MT3: *Paracentrotus lividus* MT [23], Sp_MTb: *Strongylocentrotus purpuratus* MTb (AAA30063.1), Sp_MTa: *Strongylocentrotus purpuratus* MTa (AAA30061.1), Sn_MT: *Sterechinus neumayeri* MT (CAA69912.1), Pl_MT4/MT8: *Paracentrotus lividus* MT (new annotations)

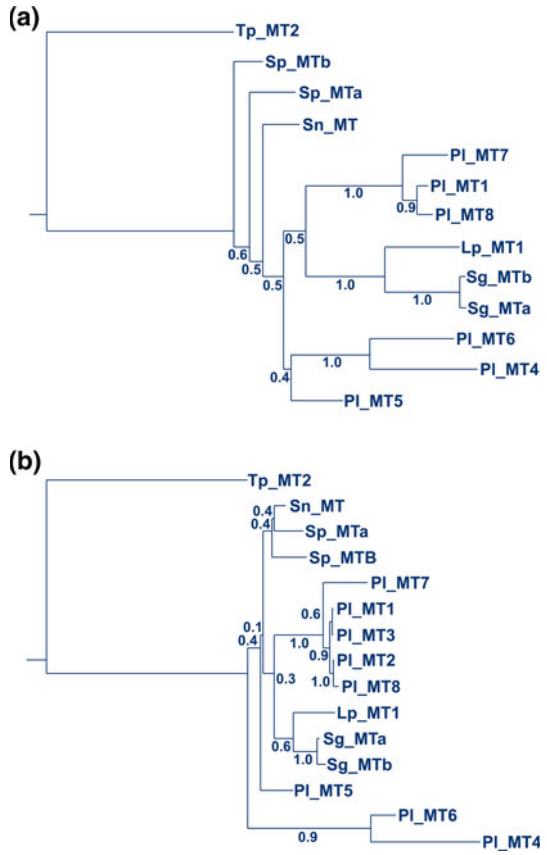

Fig. 3 **a** Nucleotide-based neighbour-joining tree of Echinodermata metallothioneins (MTs) using *Tetrahymena pyriformis* MT sequence (DQ518910.1) as outgroup. The trees are drawn with Archeopterix. Computed distance matrix in Phylip format. Number of bootstraps 1,000. Coding DNA sequences. List of bootstrap trees in Newick format. Confidence values are indicated. Nucleotide sequences and abbreviations: Sp_MTa: *S. purpuratus* metallothionein MTa (K02464.1), Sp_MTb: *S. purpuratus* metallothionein MTb (M15989.1), Lp_MT1: *L. pictus* metallothionein (LPU83401), Sn_MT1: *S. neumayeri* metallothionein (Y08621.1), Sg_MTa: *S. granularis* metallothionein (Z67877.1), Sg_MTb: *S. granularis* metallothionein (Z66530.1), Pl_MT1: *P. lividus* metallothionein MT1 (AJ310190.1), Pl_MT4/MT8: *P. lividus* MT (new annotations). **b** Amino acid sequence-based neighbor-joining tree. Substitution model: Jones-Taylor-Thornton matrix. Protein sequences and abbreviations are as indicated in the legend of Fig. 2

(here named *Pl*MT3) in the same position contains Gln. These differences are probably due to allelic polymorphism.

Nucleotide coding and deduced aminoacid sequences were aligned together with Echinodermata sequences retrieved from GenBank, using ClustalW2 (see Fig. 2 for aminoacid sequence alignment) and phylogenetic trees were constructed with PHYLIP software (neighbor-joining clustering method using *Tetrahymena pyriformis* MT sequence as outgroup). As expected, *Pl*MT1, *Pl*MT2, *Pl*MT3, and *Pl*MT8 are in the same branch of the tree and they are together with *Pl*MT7. *Pl*MT4 and *Pl*MT6 are the most divergent with respect to other sea urchin MT sequences (Fig. 3a, b).

The *Pl*MT family members, identified here, have the pattern of cysteine arrangement in the sequence that is peculiar of sea urchin MTs and that follows an order opposite to that of mammalian MTs, with the cysteine doublets located in the N-terminal and not in the C-terminal moiety [17]. *Pl*MT4, *Pl*MT7 and *Pl*MT8 do not respect the beginning of sequence pattern (P-D–x–K–C–[V,F]–C–C–x(5)–C–x–C–x(4)–C–C–x(4)–C–C–x(4,6)–C–C located near the N-term) that should characterize Echinodermata MTs. Moreover, *Pl*MT4 and *Pl*MT6 have a very divergent second structural domain. Finally, *Pl*MT5 and *Pl*MT6 are respectively 57 and 61 aa long, shorter than the expected length for Echinodermata family MTs (http://www.bioc.uzh.ch/mtpage/classif.html) [24, 25]. Therefore, given that in the previous MT classification only the two *S. purpuratus* MT sequences known at that time were taken into account, now a new classification including new MT sequence features could be possible.

Gene expression analysis

In this paper we report results on *Pl*MT genes we selected for validation since it is known that MT synthesis is an early response event in Cd treatment.

To elucidate potential changes in different *Pl*MTs mRNA expression levels, we performed reverse transcription quantitative PCR experiments on RNA extracted from embryos treated with 100 μM $CdCl_2$ for 30 h and from control embryos. RT-qPCR experiments showed that in control embryos, *Pl*MT4, *Pl*MT5 and *Pl*MT6 RNA levels are very low (the genes are virtually not expressed), in fact the threshold cycle mean for these targets is >30. Differently, *Pl*MT7 and PlMT8 mRNA levels are higher (threshold cycle mean 22.7 and 23.4 respectively). The 18S rRNA was used to normalize each sample for variation in RNA loading and, referring to 18S rRNA quantity, we could calculate the starting quantity in treated and control embryos in arbitrary units (1 AU = 18S rRNA/1,000).

RT-qPCR results showed the quantity of mRNA transcribed from constitutively active MT genes was significantly increased in Cd-treated embryos (*Pl*MT7 3.8×, *Pl*MT8 3.3×), and moreover, that mRNA levels of inducible *Pl*MT genes reached the basal mRNA level achieved by the constitutive MT genes.

Therefore, RT-qPCR experiments show the specific activation of *Pl*MT4, *Pl*MT5 and *Pl*MT6 putative inducible genes and the upregulation (or mRNA stabilization) of the so called constitutive *Pl*MT7 and *Pl*MT8 genes (Fig. 4a).

MT specificity analysis

Different Cd concentrations induce different survival responses and toxic effects in sea urchin embryos [8],

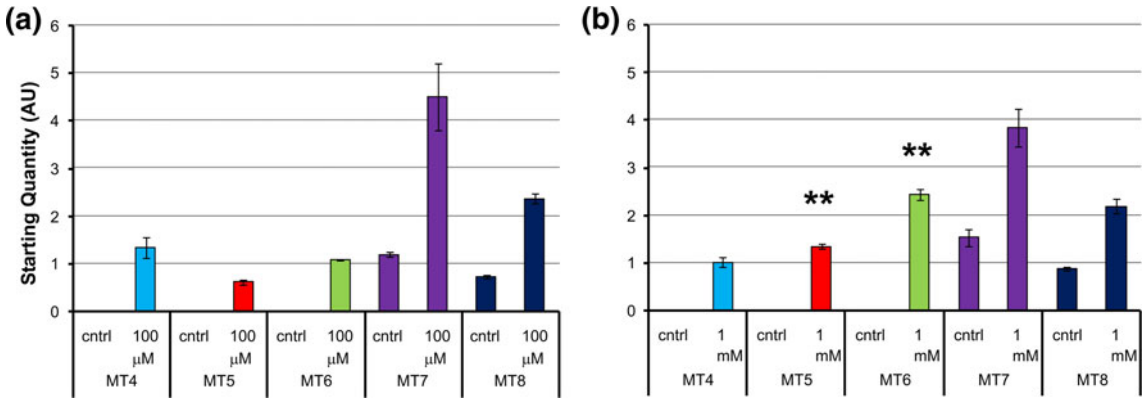

Fig. 4 Cadmium-induced alterations in MT gene expression. RT-qPCR results showing *P. lividus* MT mRNA levels, with respect to 18S rRNA in arbitrary units (AU). **a** 100 µM $CdCl_2$ treatment up to 30 hpf (hours post fertilization). **b** 1 mM $CdCl_2$ treatment up to 24 hpf. 1 AU = 18S rRNA/1,000. *Bars* represent mean ± SD, and *asterisks* denote responses that are significantly different from the 100 µM $CdCl_2$ treatment (**$P < 0.05$)

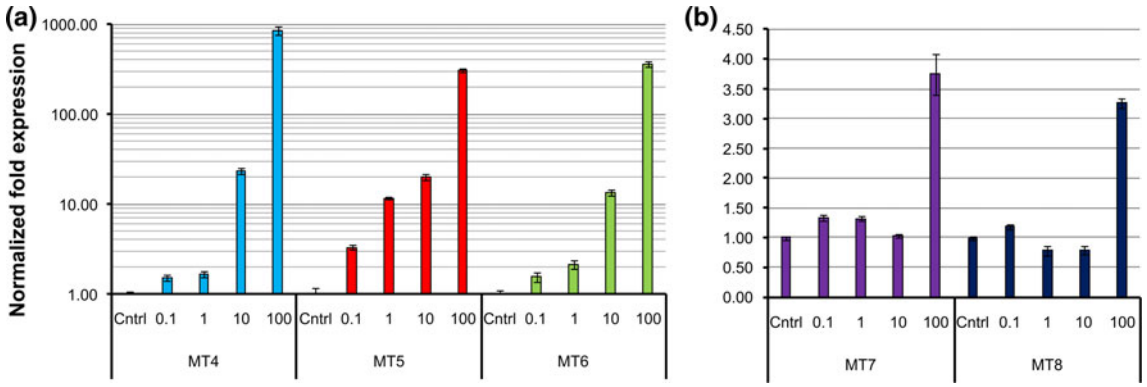

Fig. 5 RT-qPCR results showing *P. lividus* MT mRNA levels as relative fold expression (control mRNA quantity = 1) at 30 hpf, upon treatment with 0.1, 1, 10,100 µM $CdCl_2$. **a** Induced genes; logarithmic scale. **b** Constitutive genes; linear scale. *Bars* represent mean ± SD

which is why we wanted to examine if there was a different Cd concentration threshold for the activation of the different *Pl*MT genes. In order to investigate the threshold for the activation of different *Pl*MT isogenes, we checked the variations of the different transcript levels in embryos exposed to different doses of Cd.

We treated embryos with different Cd doses (0.1, 1, 10, 100 µM $CdCl_2$) for 30 h. RT-qPCR experiments were performed with RNA extracted from treated and control embryos using specific pair of primers for each isogene. The relative abundance of mRNAs encoding MTs was calculated using the comparative ΔΔC(t) method.

Results showed that *Pl*MT5 is activated at a very low Cd concentration (0.1 µM $CdCl_2$). Besides, *Pl*MT4 and *Pl*MT6 mRNA levels rose at a relatively high Cd concentration (1–10 µM $CdCl_2$) (Fig. 5a). On the contrary, *Pl*MT7 and *Pl*MT8 genes increased their expression only in embryos treated with the highest concentration of 100 µM $CdCl_2$, whereas their mRNA levels seemed to slightly decrease at lower Cd concentrations, when mRNA levels of the other induced gene increased (Fig. 5b).

In order to deeply analyze metallothionein gene regulation, we performed RT-qPCR experiments also on RNA extracted from embryos treated with ten fold higher cadmium concentration (1 mM $CdCl_2$). Cultures were harvested at 24 h post fertilization because it is known that embryos do not survive until 30 h in the presence of such high Cd concentration [8].

RT-qPCR results showed, once again, that *Pl*MT4, *Pl*MT5 and *Pl*MT6 mRNA levels were induced by this "heavy" Cd treatment and particularly that, whereas *Pl*MT4 mRNA level remained quite constant with respect to that of 100 µM $CdCl_2$ treated embryos, *Pl*MT5 and *Pl*MT6 mRNA levels further increased in response to 1 mM $CdCl_2$ treatment. Differently, *Pl*MT7 and *Pl*MT8 (constitutive genes) mRNA levels remained quite constant or slightly decreased with respect to the 100 µM $CdCl_2$ treated embryos (Fig. 4b).

Discussion

Metazoan genomes contain large numbers of genes that participate in responses to environmental stressors. A specific set of genes comprises the "chemical defensome". The chemical defensome is an integrated network of genes and pathways that allows an organism to mount an orchestrated defense against toxic chemicals, including exogenous compounds (xenobiotics) and endogenously produced toxicants, such as free radicals.

The produced proteins include enzymes that transform chemicals to metabolites less toxic and that are more readily excreted, efflux transporters that actively eliminate toxicants and transformed products, antioxidant enzymes protecting against externally and internally generated ROS or other radicals, and soluble receptors and ligand-activated transcription factors that act as sensors of toxicants or cellular damage [26–28].

Almost all of the gene families or superfamilies contributing to chemical defense in vertebrates are also represented in the sea urchin. Similarly, many of the gene families or superfamilies that participate in the defensome in deuterostomes are found in protostomes, suggesting the origin of an orchestrated chemical defensome in a bilaterian ancestor.

In sea urchin *Strongylocentrotus purpuratus* more than half of the defense genes are expressed during embryonic or larval life stages, indicating their importance during development. Gene products that protect against injury from chemicals are especially important in embryos, given the complex chemical signaling pathways governing development as well as the need to protect the genome of the germ cells. Moreover, at the earliest stages of sea urchin development each cell must carry out all of the protective functions typically carried out by multiple cells or tissues in an adult, therefore chemical defense genes may be especially critical for resilience and survival of these early stages [29, 30].

Consequently the sea urchin is an excellent model for studying the problem of cellular defense during embryonic development, thanks also to the technologies established for gene expression manipulation (e.g., control of transcription factor expression) and to the extensive work done to determine gene regulatory networks [31].

Previously, it has been shown that *Paracentrotus lividus* sea urchin embryos developed in 1 mM $CdCl_2$ undergo irreversible inhibition of development or severe malformations, whereas sub lethal Cd doses (10–100 μM $CdCl_2$) are able to activate different molecular responses (HSPs and MTs synthesis, autophagic or apoptotic processes) that can allow embryo survival (for review, see [8]).

We studied in detail the early defense strategies activated by *P. lividus* 30 h embryos, in response to exposure to sub lethal doses of Cd (100 μM $CdCl_2$). As it is known that, a 30 h treatment with 100 mM $CdCl_2$ is sufficient enough to induce detoxification processes and defense strategies, but, at the same time, does not have harmful effects on embryo development [8–11, 15, 16], we chose this kind of treatment to analyze the induced transcripts and to compare it to that of control (untreated) embryos by SSH technique.

Our results indicate that under these conditions *P. lividus* embryos activated, like *S. purpuratus* embryos [27], a chemical defensome response. We found in fact an up regulation of five metallothionein genes, of a selenophosphate synthase gene and of a number of genes coding for different oxidoreductases. This latter finding suggesting a putative involvement of oxidative phosphorylation components is very interesting. In fact the activation of cytochromeB and cytochrome oxidaseI genes and the down regulation of NADH dehydrogenase subunit genes, together with the activation of the inhibitor of nitric oxide synthase gene, are indicative of "metabolic hypoxia" and redox stress and a tentative of ROS reduction [32, 33].

Furthermore, we would like to remember that metal detoxification is a central part of the chemical defensome strategy, and MTs are the proteins that play a major role in metal homeostasis acting as metal reservoirs.

Nemer et al. [17, 36] isolated in *S. purpuratus* two MT isoforms that were termed MTa and MTb. MTb is coded by at least two genes that does not differ in their coding sequence. *Sp*MTA and *Sp*MTB_1 genes have been shown to have different temporal and spatial expressions during embryogenesis. Their expression is enhanced by treatment of the embryos with Zn and Cd ions and inhibited after heavy-metal depletion by the addition of a chelator. They also play roles in nutrient metal homeostasis and in apoptosis [18, 34–36].

In *P. lividus* embryos only one MT gene had been previously identified which is constitutively expressed at basal levels in eggs and during embryogenesis and whose expression is increased by sub-lethal Cd concentrations treatment [12].

By SSH technique, we identified and isolated five paralogous MT isogenes and we studied their expression in the *P. lividus* sea urchin embryo, assessing their Cd-dependent transcriptional activation by reverse transcription and Real Time PCR. We found that two metallothionein isogenes (*Pl*MT7 and *Pl*MT8) appeared to be constitutively expressed and upregulated upon Cd treatment, whereas three other isogenes (*Pl*MT4, *Pl*MT5, *Pl*MT6) were not transcribed in control embryos and were specifically activated in response to Cd treatment.

In particular, given that it is known that different Cd concentrations induce different survival responses and toxic effects in sea urchin embryos [8], we investigated the Cd concentration threshold for the activation of the different five isogenes. In particular we checked the variations of transcript levels in embryos exposed to different doses

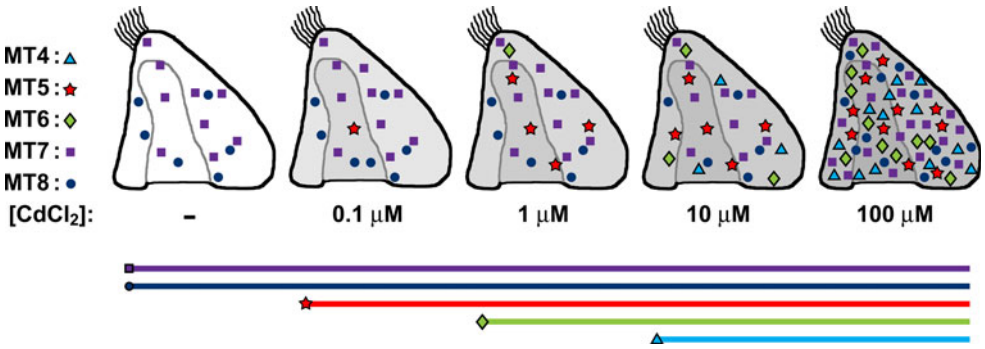

Fig. 6 Cartoon showing the MT gene expression pattern after embryo was treated with different cadmium concentrations. The *grey* level is virtually proportional to the accumulated cadmium amount and the number of each symbol is roughly proportional to each mRNA level. *Bars* at the *bottom* show up MT mRNA differential presence following different cadmium exposure

of Cd by RT-qPCR experiments. We found that, in spite of the presence of MT constitutive transcripts (*Pl*MT7 and *Pl*MT8) that are up regulated until reaching a plateau (see results of RT-qPCR of 100 µM and 1 mM treatments in Fig. 4), the Cd-treated embryos induced other MT isogenes (*Pl*MT4, *Pl*MT5 and *Pl*MT6) suggesting that the Cd response involves preferential activation of specific isogenes. We found also that, increasing Cd concentration, different isogenes are involved in a different and hierarchical way: first *Pl*MT5 then *Pl*MT4 and *Pl*MT6. *Pl*MT7 and *Pl*MT8 are involved only at higher Cd concentrations. Furthermore, we should remark that the transcript levels of *Pl*MT7 and *Pl*MT8 isogenes not only do not increase at low Cd concentrations (when mRNA levels of the other induced isogenes increase) but seem to slightly decrease, again suggesting different function for the different isogenes (see cartoon in Fig. 6).

Conclusion

Our results confirm that *P. lividus* is able to activate a "classical" defense response as other deuterostoma [30], and that this response initially involves the expression of MT genes. In fact, we identified five different transcripts coding for five MT isotypes having the peculiar cysteine arrangement found in *Sp*MTs [17, 24, 25]. Inspection of the protein sequence features allowed us to identify other peculiar properties that are characteristic of the single MT family member. This may allow a revision of the sea urchin MT classification method [24, 25] and above all is in agreement with the existence of functional MT specific isoforms probably involved differentially in development and/or detoxification [37–43].

Moreover—for the first time—our findings highlight a hierarchical detoxification response carried out by the differential transcriptional induction of each MT gene. These latter findings, in our opinion, are very interesting since heavy metals are able to hinder gametogenesis, suppress embryogenesis and hamper development in aquatic organism. Moreover, an MT physiological function that make possible a correct developmental program has been reviewed [44]. From this point of view, the hierarchical induction of the different *Pl*MT isogenes we found, together with the activation of autophagic and then apoptotic pathways [8, 15], is remarkable and can contribute to the higher survival rate of larvae under different environmental conditions.

Finally, although the main aim of this study was to investigate the molecular response of *P. lividus* embryos that are subjected to Cd treatment and how this can affect the developmental pathways, certainly, potential applications of our results may be in the future to establish the sea urchin as a test system for cadmium pollution in marine environments. Indeed, the differential induction of MTs we have found, could be used as a molecular differential biomarker for toxicity tests, and their quantification could be a new method of evaluating marine water from contaminated sites.

Acknowledgments We would like to thank C. Luparello and V. Matranga for their critical reading and feedbacks on this manuscript. We would also like to apologize with all our colleagues whose work was not properly cited due to space restriction. This work was supported by MIUR (ex 60 %) grant to F.G.

References

1. Rainbow PS (2002) Kenneth Mellanby Review Award. Trace metal concentrations in aquatic invertebrates: why and so what? Environ Pollut 120:497–507
2. Viarengo A (1989) Heavy metal in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. Aquat Sci 1:295–317
3. Wang Z, Yan C, Kong H, Wu D (2010) Mechanisms of cadmium toxicity to various trophic saltwater organisms. In: Parvau RG (ed) Cadmium in the environment. Nova Science Publishers Inc, New York, pp 297–336
4. Au DW, Lee CY, Chan KL, Wu RS (2001) Reproductive impairment of sea urchins upon chronic exposure to cadmium. Part I: effects on gamete quality. Environ Pollut 111:1–9
5. Matranga V, Toia G, Bonaventura R, Müller WE (2000) Cellular and biochemical responses to environmental and experimentally induced stress in sea urchin coelomocytes. Cell Stress Chaperones 5:113–120

6. Matranga V, Pinsino A, Celi M, Natoli A, Bonaventura R, Schröder HC, Müller WEG (2005) Monitoring chemical and physical stress using sea urchin immune cells. Prog Mol Subcell Biol 39:85–110
7. Radenac G, Fichet D, Miramand P (2001) Bioaccumulation and toxicity of four dissolved metals in *Paracentrotus lividus* sea-urchin embryo. Mar Environ Res 51:151–166
8. Roccheri MC, Matranga V (2010) Cellular, biochemical and molecular effects of cadmium on marine invertebrates: focus on *Paracentrotus lividus* sea urchin development. In: Parvau RG (ed) Cadmium in the environment. Nova Science Publishers Inc, New York, pp 337–366
9. Agnello M, Filosto S, Scudiero R, Rinaldi AM, Roccheri MC (2007) Cadmium induces an apoptotic response in sea urchin embryos. Cell Stress Chaperones 12:44–50
10. Filosto S, Roccheri MC, Bonaventura R, Matranga V (2008) Environmentally relevant cadmium concentrations affect development and induce apoptosis of *Paracentrotus lividus* larvae cultured in vitro. Cell Biol Toxicol 24:603–610. doi:10.1007/s10565-008-9066-x
11. Roccheri MC, Agnello M, Bonaventura R, Matranga V (2004) Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochem Biophys Res Commun 321:80–87. doi:10.1016/j.bbrc.2004.06.108
12. Russo R, Bonaventura R, Zito F, Schröder HC, Müller I, Müller WEG, Matranga V (2003) Stress to cadmium monitored by metallothionein gene induction in *Paracentrotus lividus* embryos. Cell Stress Chaperones 8:232–241
13. Schröder HC, Di Bella G, Janipour N, Bonaventura R, Russo R, Müller WEG, Matranga V (2005) DNA damage and developmental defects after exposure to UV and heavy metals in sea urchin cells and embryos compared to other invertebrates. Prog Mol Subcell Biol 39:111–137
14. Amiard J-C, Amiard-Triquet C, Barka S, Pellerin J, Rainbow PS (2006) Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat Toxicol 76:160–202. doi:10.1016/j.aquatox.2005.08.015
15. Chiarelli R, Agnello M, Roccheri MC (2011) Sea urchin embryos as a model system for studying autophagy induced by cadmium stress. Autophagy 7:1028–1034
16. Geraci F, Pinsino A, Turturici G, Savona R, Giudice G, Sconzo G (2004) Nickel, lead, and cadmium induce differential cellular responses in sea urchin embryos by activating the synthesis of different HSP70s. Biochem Biophys Res Commun 322:873–877. doi:10.1016/j.bbrc.2004.08.005
17. Nemer M, Wilkinson DG, Travaglini EC, Sternberg EJ, Butt TR (1985) Sea urchin metallothionein sequence: key to an evolutionary diversity. Proc Natl Acad Sci USA 82:4992–4994
18. Nemer M, Thornton RD, Stuebing EW, Harlow P (1991) Structure, spatial, and temporal expression of two sea urchin metallothionein genes, SpMTB1 and SpMTA. J Biol Chem 266:6586–6593
19. Gianguzza F, Di Bernardo MG, Sollazzo M, Palla F, Ciaccio M, Carra E, Spinelli G (1989) DNA sequence and pattern of expression of the sea urchin (*Paracentrotus lividus*) alpha-tubulin genes. Mol Reprod Dev 1:170–181
20. Gianguzza F, Di Bernardo MG, Fais M, Palla F, Casano C, Russo R, Spinelli G (1990) Sequence and expression of *Paracentrotus lividus* alpha tubulin gene. Nucleic Acids Res 18:4915
21. Casano C, Ragusa M, Cutrera M, Costa S, Gianguzza F (1996) Spatial expression of alpha and beta tubulin genes in the late embryogenesis of the sea urchin *Paracentrotus lividus*. Int J Dev Biol 40:1033–1041
22. Casano C, Roccheri MC, Onorato K, Cascino D, Gianguzza F (1998) Deciliation: a stressful event for *Paracentrotus lividus* embryos. Biochem Biophys Res Commun 248:628–634
23. Scudiero R, Capasso C, Del Vecchio-Blanco F, Savino G, Capasso A, Parente A, Parisi E (1995) Isolation and primary structure determination of a metallothionein from *Paracentrotus lividus* (Echinodermata, Echinoidea). Comp Biochem Physiol B 111:329–336
24. Binz PA, Kägi JHR (1999) Metallothionein: molecular evolution and classification. In: Klaassen C (ed) Metallothionein IV. Birkhäuser Verlag, Basel, pp 7–13
25. Capdevila M, Atrian S (2011) Metallothionein protein evolution: a miniassay. J Biol Inorg Chem 16:977–989. doi:10.1007/s00775-011-0798-3
26. de Torres M, Sanchez P, Fernandez-Delmond I, Grant M (2003) Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in RPM1-mediated resistance. Plant J 33:665–676
27. Goldstone JV, Hamdoun A, Cole BJ, Howard-Ashby M, Nebert DW, Scally M, Dean M, Epel D, Hahn ME, Stegeman JJ (2006) The chemical defensome: environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome. Dev Biol 300:366–384. doi:10.1016/j.ydbio.2006.08.066
28. Goldstone JV (2008) Environmental sensing and response genes in cnidaria: the chemical defensome in the sea anemone Nematostella vectensis. Cell Biol Toxicol 24:483–502. doi:10.1007/s10565-008-9107-5
29. Epel D (2003) Protection of DNA during early development: adaptations and evolutionary consequences. Evol Dev 5:83–88
30. Hamdoun A, Epel D (2007) Embryo stability and vulnerability in an always changing world. Proc Natl Acad Sci USA 104(6):1745–1750. doi:10.1073/pnas.0610108104
31. Damle SS, Davidson EH (2012) Synthetic in vivo validation of gene network circuitry. Proc Natl Acad Sci USA 109(5):1548–1553. doi:10.1073/pnas.1119905109
32. Moncada S, Erusalimsky JD (2002) Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat Rev Mol Cell Biol 3:214–220. doi:10.1038/nrm762
33. Xu W, Charles IG, Moncada S (2005) Nitric oxide: orchestrating hypoxia regulation through mitochondrial respiration and the endoplasmic reticulum stress response. Cell Res 15:63–65. doi:10.1038/sj.cr.7290267
34. Angerer LM, Kawczynski G, Wilkinson DG, Nemer M, Angerer RC (1986) Spatial patterns of metallothionein mRNA expression in the sea urchin embryo. Dev Biol 116:543–547
35. Vasák M, Hasler DW (2000) Metallothioneins: new functional and structural insights. Curr Opin Chem Biol 4:177–183
36. Wilkinson DG, Nemer M (1987) Metallothionein genes MTa and MTb expressed under distinct quantitative and tissue-specific regulation in sea urchin embryos. Mol Cell Biol 7:48–58
37. Capdevila M, Bofill R, Palacios Ò, Atrian S (2012) State-of-the-art of metallothioneins at the beginning of the 21st century. Coord Chem Rev 256:46–62. doi:10.1016/j.ccr.2011.07.006
38. Guirola M, Naranjo Y, Capdevila M, Atrian S (2011) Comparative genomics analysis of metallothioneins in twelve *Drosophila* species. J Inorg Biochem 105:1050–1059. doi:10.1016/j.jinorgbio.2011.05.004
39. Höckner M, Stefanon K, de Vaufleury A, Monteiro F, Pérez-Rafael S, Palacios O, Capdevila M, Atrian S, Dallinger R (2011) Physiological relevance and contribution to metal balance of specific and non-specific Metallothionein isoforms in the garden snail *Cantareus aspersus*. Biometals 24:1079–1092. doi:10.1007/s10534-011-9466-x
40. Thirumoorthy N, Shyam Sunder A, Manisenthil Kumar K, Senthil Kumar M, Ganesh G, Chatterjee M (2011) A review of metallothionein isoforms and their role in pathophysiology. World J Surg Oncol 9:54. doi:10.1186/1477-7819-9-54
41. Soazig L, Marc L (2003) Potential use of the levels of the mRNA of a specific metallothionein isoform (MT-20) in mussel (*Mytilus*

edulis) as a biomarker of cadmium contamination. Mar Pollut Bull 46:1450–1455. doi:10.1016/S0025-326X(03)00283-2
42. Wang W-X, Rainbow PS (2010) Significance of metallothioneins in metal accumulation kinetics in marine animals. Comp Biochem Physiol C 152:1–8. doi:10.1016/j.cbpc.2010.02.015
43. Zeitoun-Ghandour S, Charnock JM, Hodson ME, Leszczyszyn OI, Blindauer CA, Stürzenbaum SR (2010) The two *Caenorhabditis elegans* metallothioneins (CeMT-1 and CeMT-2) discriminate between essential zinc and toxic cadmium. FEBS J 277(11):2531–2542. doi:10.1111/j.1742-4658.2010.07667.x
44. Mao H, Wang D-H, Yang W-X (2012) The involvement of metallothionein in the development of aquatic invertebrate. Aquat Toxicol 110–111:208–213. doi:10.1016/j.aquatox.2012.01.018