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RESEARCH REPORT

Molecular characterization of two glutathione peroxidase genes in *Mytilus galloprovincialis* and their transcriptional responses to sub-chronic arsenate and cadmium exposure

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Abstract

Glutathione peroxidases (GPxs) are key enzymes in the antioxidant defense system of living organisms, and protect organisms against oxidative stresses. In this study, the full-length cDNA sequences encoding cytosolic GPx (MgcGPx) and phospholipid-hydroperoxide GPx (MgGPx4) were identified from *Mytilus galloprovincialis*. The mussels were exposed to 0, 1, 10, and 100 μg/L cadmium and arsenate for 30 days. The mRNA transcripts of these two genes and total GPx activity were examined in the gills and digestive gland after contaminants exposure. The mussels exposed to cadmium and arsenate responded mainly by down-regulating MgcGPx and MgGPx4 mRNA transcription in gills and up-regulating transcription in digestive gland. However, total GPx activities increased following cadmium exposure but decreased after arsenate stress in both tissues. These results suggest that MgcGPx and MgGPx4 perhaps play an important role in maintaining cellular redox homeostasis and protecting *M. galloprovincialis* against cadmium and arsenate toxicity. It can also be inferred that these genes have the potential to be used as molecular biomarkers for assessing cellular stress and toxicity of contaminants in this mussel.

Key Words: cytosolic GPx; phospholipid-hydroperoxide GPx; arsenic; cadmium; bivalve; antioxidant enzyme

Introduction

In recent years, a great number of contaminants including metals, petroleum oils and organic pollutants have been discharged into the Bohai Sea, a territorial sea in North China (Ma *et al*., 2001). Among these contaminants, metal contaminants have been recognized as the major pollutants in coastal areas. Cadmium (Cd) has been found in high concentrations in marine environments of the Bohai Sea (Meng *et al*., 2004; Meng *et al*., 2008; Wang *et al*., 2009). In addition, arsenic (As) pollution also has been of great concern because a total arsenic concentration was up to 440 μg L^{-1} in some polluted estuarine areas of the Bohai Sea (Meng *et al*., 2004; Wu *et al*., 2013).

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The pollutants including metal contaminants constitute a potential threat to marine organisms (Winston and Di Giulio, 1991). The toxic damage induced by metals and arsenic is generally associated with excessive formation of reactive oxygen species (ROS), which cause oxidative modification of the major cellular macromolecules (Leonard *et al*., 2004; Ventura-Lima *et al*., 2011). Both Cd and arsenic have been shown to be toxic to marine bivalves and affect their physiological status by causing oxidative stress (Dovzhenko *et al*., 2005; Wang *et al*., 2010; Chakraborty *et al*., 2010). Excessive ROS production has been found to oxidize and damage cell membrane, proteins, and nucleic acids (Hensley *et al*., 2000). In marine animals, the oxidative stress can be counteracted by non-enzymatic antioxidant and enzymatic antioxidant systems (Fernández *et al*., 2010). The key components of these enzymatic antioxidant systems include glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) (Apel and Hirt, 2004).

GPx (EC 1.11.1.9 and EC 1.11.1.12) can

catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) (Margis, 2008). They are generally classified into two subgroups as selenium-dependent GPx and non-selenium GPx, based on the presence of selenocysteine (Sec) encoded by a stop codon TGA (Arthur, 2000). At present, eight GPx isozymes have been characterized in mammals, including: cytosolic GPx (GPx1), gastrointestinal GPx (GPx2), plasma GPx (GPx3), phospholipid hydroperoxide GPx (GPx4), epididymal GPx (GPx5), olfactory epithelium GPx (GPx6), endoplasmic reticulum phospholipid hydroperoxide GPxs (GPx7 and GPx8) (Herbette *et al*., 2007; Nguyen *et al*., 2011). In contrast to other tetrameric GPxs, GPx4 is monomeric and membrane-associated, especially in membrane fractions. GPx4 is unique in its substrate specificity because it can interact with lipophilic substrates, including the peroxidized phospholipids and cholesterol, and reduce these hydroperoxides to hydroxide compounds (Thomas *et al*., 1990). They mainly participate in the repair of disrupted bio-membranes, which has been considered as the main defense strategy against oxidative membrane damage (Bae *et al*., 2009).

The role of cytosolic selenium-dependent GPxs in host immune and antioxidant defense system has been well documented in many mollusks, such as *Unio tumidus* (Doyen *et al*., 2006), *Crassostrea gigas* (Jo *et al*., 2008), *Chlamys farreri* (Mu *et al*., 2010), *Meretrix meretrix* (Wang *et al*., 2011a), *Venerupis philippinarum* (Zhang *et al*., 2011; Zhang *et al*., 2012) and *Haliotis discus hannai* (Wu *et al*., 2010). However, GPx4s have been less characterized and their roles in antioxidant system are less studied in aquatic animals. Until recently, only a few GPx4 genes have been cloned in aquatic organisms such as hydra (Dashe *et al*., 2006), midge (Nair *et al*., 2012) and several fishes (Wang *et al*., 2012; Pacitti *et al*., 2013). To our knowledge, no GPx4 gene has been cloned from mollusks although GPx4 activity was observed in the mussel *Perna perna* (Almeida *et al*., 2005).

The mussel *Mytilus galloprovincialis*, extensively dispersed in the Bohai Sea, has a high adaptive ability to environments and occupies a broad range of habitat types. They are apt to be exposed to pollutants and suffer from oxidative stress in esutarine and coastal area of the Bohai sea. In China, this mussel has been used as a biomonitoring organism to assess the marine environment. Therefore, it is necessary to study how the antioxidant system of the mussel responds to environmental stress. To investigate the possible role of GPxs in protecting marine mussel from oxidative damage, two GPx genes were characterized and their transcriptional responses to sub-chronic cadmium and arsenic exposures were studied. In addition, the possibility of these genes used as biomarkers was also discussed.

Materials and methods

Animal culture

Adult animals (shell length: 5.0 - 7.0 cm) were purchased from a local mussel culture farm and acclimatized in aerated seawater (33 psu) at 20 °C for 7 days before commencement of the exposure experiment. During the acclimatization period, the mussels were fed with *Phaeodactylum tricornutum* and *Platymonas helgolandica*, and the seawater was completely renewed once every 24 h.

Contaminant exposure

After the acclimatization, the mussels were divided into 21 groups and cultured in polyethylene tanks with 20 L seawater, each containing 20 individuals. The exposure experiments were performed using analytical grade salts of $CdCl_2{\cdot}2.5H_2O$ and $Na_2HAsO_4{\cdot}12H_2O$ (Guoyao, Shanghai, China). Stock solutions were prepared in deionized water at a concentration of 1 g L^{-1}, which was high enough to prevent weighing errors and salinity change. The exposure experiment for each contaminant included three concentrations: 1, 10 and 100 μg L^{-1} (contaminant concentration, not salt concentrations). There were 3 replicates (tanks) per treatment and the other three untreated tanks were employed as the control groups. The mussels were fed as described above and the culture medium was renewed once every 24 h. The gills and digestive glands of four individuals from each tank were randomly sampled for gene expression and antioxidant enzyme activity analysis after 30 days of exposure respectively. All the samples were quickly frozen in liquid nitrogen and then transferred to a -80 °C refrigerator till use.

RNA isolation, cDNA synthesis and cloning of mussel GPx genes

Total RNA was extracted from gills using the TRIzol Reagent (Invitrogen, USA) following the supplier's protocol. The extracted RNA was then treated with RQ1 RNase-Free DNase (Promega, USA) to remove DNA contamination. Single-stranded cDNAs were synthesized from one microgram of the total RNA using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. BLAST analysis of all expressed sequence tag (EST) sequences from a cDNA library of *M. gallopronvicialis* (unpublished) revealed that two ESTs were highly similar to the previously identified GPx genes, respectively. The 3′ ends of MgcGPx and MgGPx4 were obtained by rapid amplification of cDNA ends (RACE) using the SMART™ RACE cDNA amplification Kit (Clontech, USA) according to the manufacturer's manual. Two RACE primers were designed according to these ESTs and they were included in Table 1. The PCR products were purified using agarose gel electrophoresis, cloned into the pMD-18T simple vector (Takara, Japan) and then sequenced in both directions by the Chinese National Human Genome Center (SinoGenoMax, Beijing, China).

Sequence analysis

The MgcGPx and MgGPx4 cDNA sequences were analyzed using the BLASTx search program (http://www.ncbi.nlm.nih.gov/blast/). The deduced amino acid sequences were analyzed using the ExPASy server (http://www.expasy.org/tools/). Multiple alignments were performed with the ClustalW program (http://www.ebi.ac.uk/clustalw/). A

Table 1 Primers used in this study

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Description
MgcGPx	GGAAAATGGAAACGGTGAAG	TATGGGAACCTGTGACAAGAAC	3' RACE
MgGPx4	AGGAGCCTGGAACTGAAGC	GTTCTGGGGATGTCAACCTG	3' RACE
β-actin	GCTATCCAGGCCGTACTCT	GCGGTGGTTGTGAATGAG	qRT-PCR
α-tubulin	GACCACCCATACCACCCTT	CTCCGTGAGATCGACATTC	qRT-PCR
18s rRNA	TCGGATTGGTGAGACTGGAT	TGCTGCCTTCCTTGGATGT	qRT-PCR
28s rRNA	CCGAGACCGAGGATTTGCC	ACCGATTCGCCACTGACCC	qRT-PCR
ubiquitin C	ATCAACAGCGTCTCATCT	GCTCAACTTCTAGCGTAAT	qRT-PCR
EF1	GCTGGTATCTCATCTAACG	CTTCACTGTATGGTGGTTC	qRT-PCR
GAPDH	AGGGTCCAATGAAGGGTG	TTAAGAGCGATGCCAGCT	qRT-PCR
DDX	CATCAGAAGAAGGTGGCT	AACAGTTGGTCGTAGGGT	qRT-PCR
MgcGPx	AATGCCCTTGAGCATGTTCG	TGAAACTAACAGCATCGTCGC	qRT-PCR
MgGPx4	AGTCAGGAGCCTGGAACTGA	TGCCTCCTTGTTTGTGTTTG	qRT-PCR

phylogenetic tree was constructed with Mega4.1 software using the neighbor-joining (NJ) method based on the alignment (Tamura *et al*., 2007). Bootstrap analysis was used with 1000 replicates to test the relative support for the branches produced by the NJ analysis. The selenocysteine insertion sequence (SECIS) elements were searched using the SECISearch 2.19 program (http://genome.unl.edu./SECISearch.html).

Quantitative real time PCR (qRT-PCR) analysis of MgcGPx and MgGPx4 expression

In order to select appropriate internal standards for qRT-PCR analysis of gene expression in mussel exposed to cadmium and arsenic, the expressions of eight housekeeping genes, *i.e*., β-actin, α-tubulin, elongation factor 1-α (EF1), 18S rRNA, 28S rRNA, ubiquitin C, DEAD-box RNA helicase (DDX) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined. The data were analyzed with geNorm to calculate the expression stability (M values) of potential reference genes required for accurate normalization (V values) (Dang and Sun, 2011). Hemocytes, gills, digestive gland, adductor muscle, mantle and gonad of six untreated individuals were sampled to investigate the tissue-specific distribution of MgcGPx and MgGPx4 transcripts. The tissues of digestive gland and gills were selected to analyze temporal expression profile of MgcGPx and MgGPx4 challenged by Cd and As^{V}, respectively. Total RNA extraction and cDNA synthesis were performed as described above. qRT-PCR was carried out in an ABI 7500 Real-time Detection System by using the SYBR ExScript qRT-PCR Kit (Takara) as described before (Wang *et al*., 2013). The PCR amplification was carried out in a total volume of 50 μL, containing 25 μL of 2 × SYBR Green PCR Master Mix, 20 μL of the diluted cDNA, 1 μL of each of primers (10 μmol L^{-1}), and 3 μL of DEPC-treated water. The thermal profile for qPCR was 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate. Dissociation curve analysis of amplicon was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The expression level of MgcGPx and MgGPx4 was analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The qRT-PCR primers of MgcGPx and MgGPx4 are shown in Table 1.

Measurement of total GPx activity in digestive gland and gills

For assays on total GPx activity, the tissues of digestive gland and gills from six mussels were weighed and homogenized individually in 10 % (w/v) 50 mM Tris-HCl buffer (pH 7.5) using a tissue grinder (Ultra Turrax IKA T10 basic, Germany) held in an ice-water cooled bath. The homogenate was centrifuged at 10,000×*g* (4 ℃) for 30 min (Eppendorf centrifuge 5804R, Germany). Then the supernatant was collected and kept at 4 °C until used for further analysis. The total protein concentrations were determined according to the method described by Bradford (1976). Total GPx activity was measured as described before (Wang *et al*., 2010). All measurements were carried out in a Magellan plate reader (Tecan, Switzerland).

Statistical analysis

SPSS 16.0 software (SPSS Inc., USA) was used for statistical analysis. The data were checked for homogeneity of variance before analysis. The data were analyzed by one-way analysis of variance (one-way ANOVA), followed by a Tukey's post hoc test. A probability level of $p < 0.05$ was considered significant.

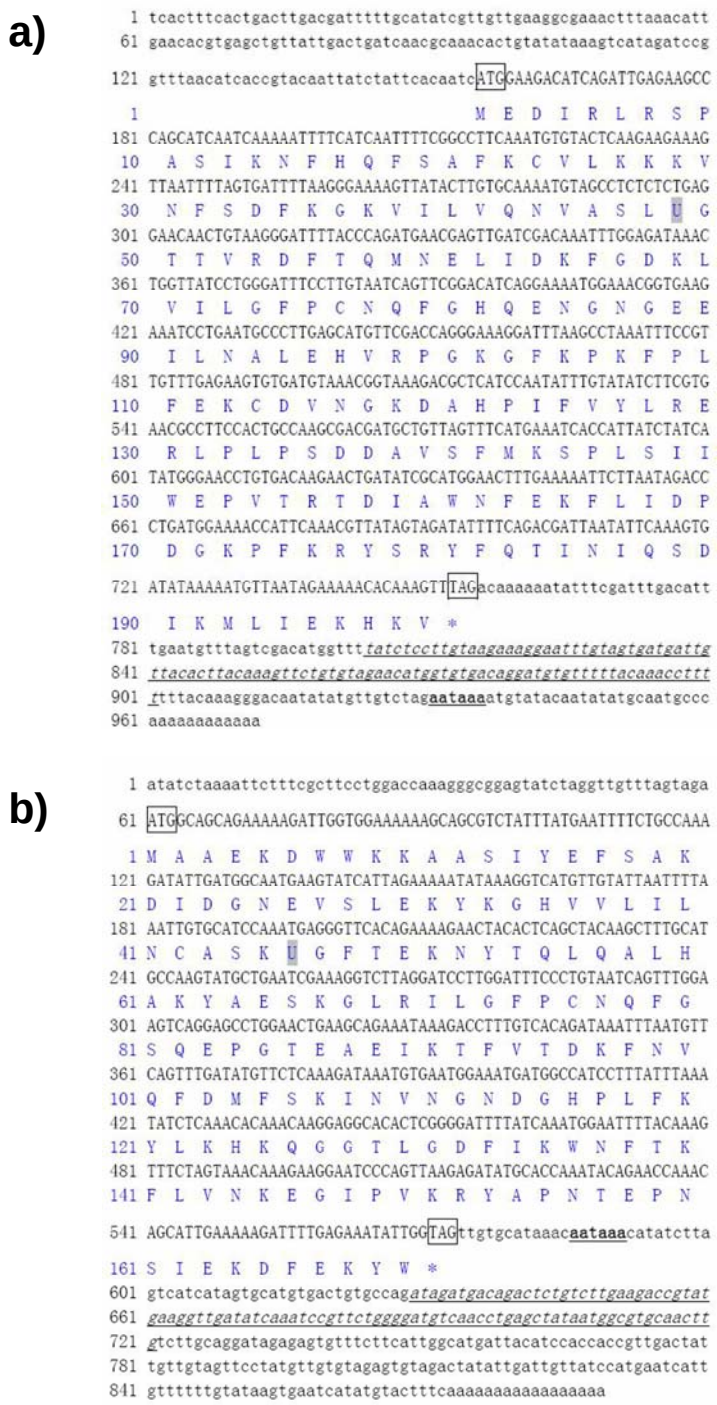

Fig. 1 The complete nucleotide and deduced amino acid sequences of MgcGPx (A) and MgGPx4 (B). The asterisk (*) indicates the stop codon. The start and stop codons are included in a box. The selenocysteine (U) is shaded. The polyadenylation signal is underlined in bold font and the predicted SECIS element is underlined in italic font.

Results

Sequence analysis

Two nucleotide sequences of 972 bp and 889 bp representing the complete cDNA sequence of MgcGPx and MgGPx4 were obtained by overlapping EST and the amplified fragments. Full-length cDNAs of MgcGPx and MgGPx4 were deposited in GenBank under the accession numbers of HQ891311 and JQ866922, respectively. The deduced amino acid sequences of MgcGPx and MgGPx4 were shown below and the corresponding nucleotide acid sequence in Figure 1.

The complete sequence of MgcGPx cDNA

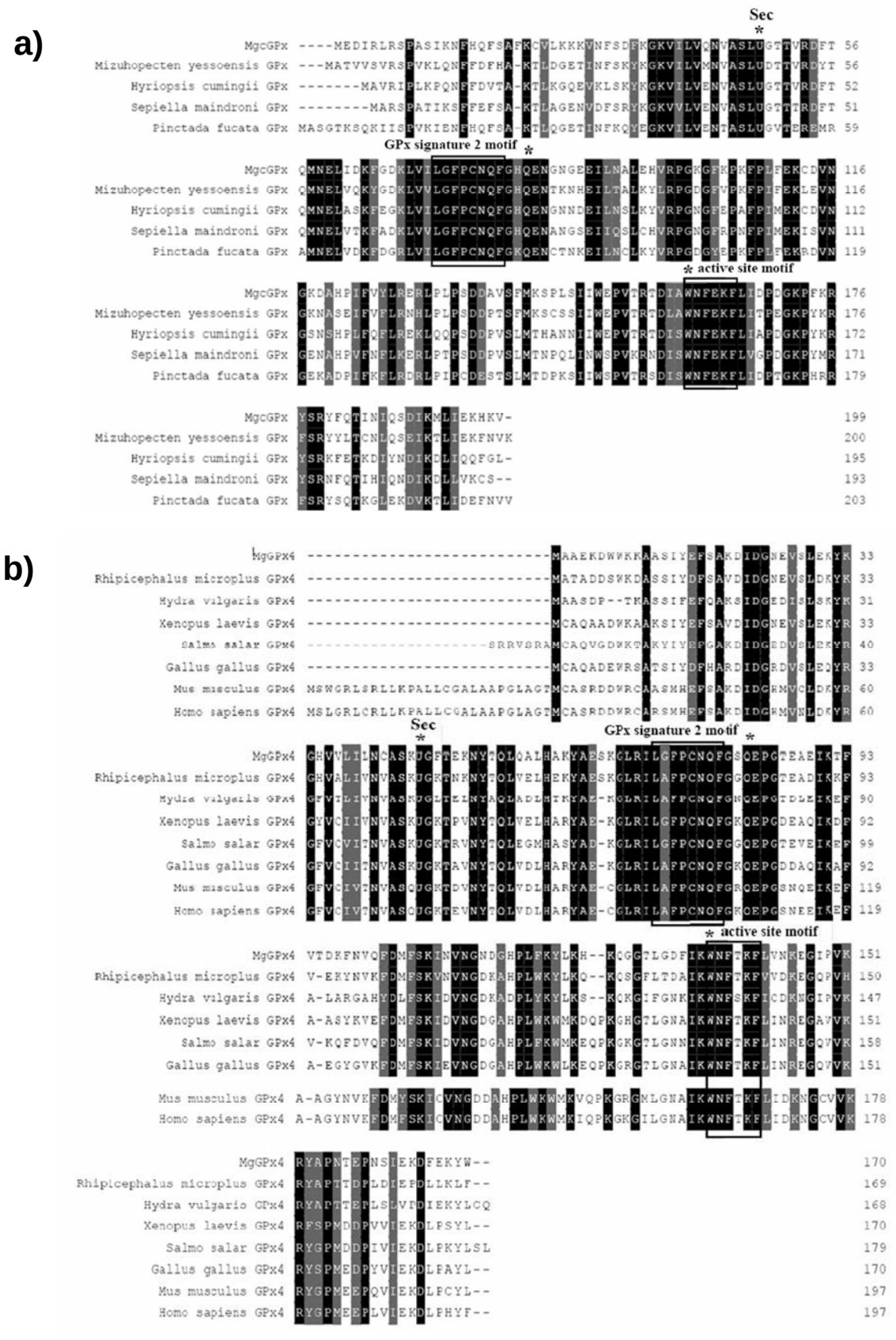

Fig. 2 Multiple sequence alignments of MgcGPx (A) and MgGPx4 (B) with other GPx orthologs deposited in GenBank. The black shadow region indicates positions where all sequences share the same amino acid residue. Gaps are indicated by dashes to improve the alignment. The GPx signature motif 2 elements and active site motifs are marked by frame, respectively. The catalytically important residues are indicated by asterisks. The GenBank accession numbers of GPx sequences are shown in Table 2.

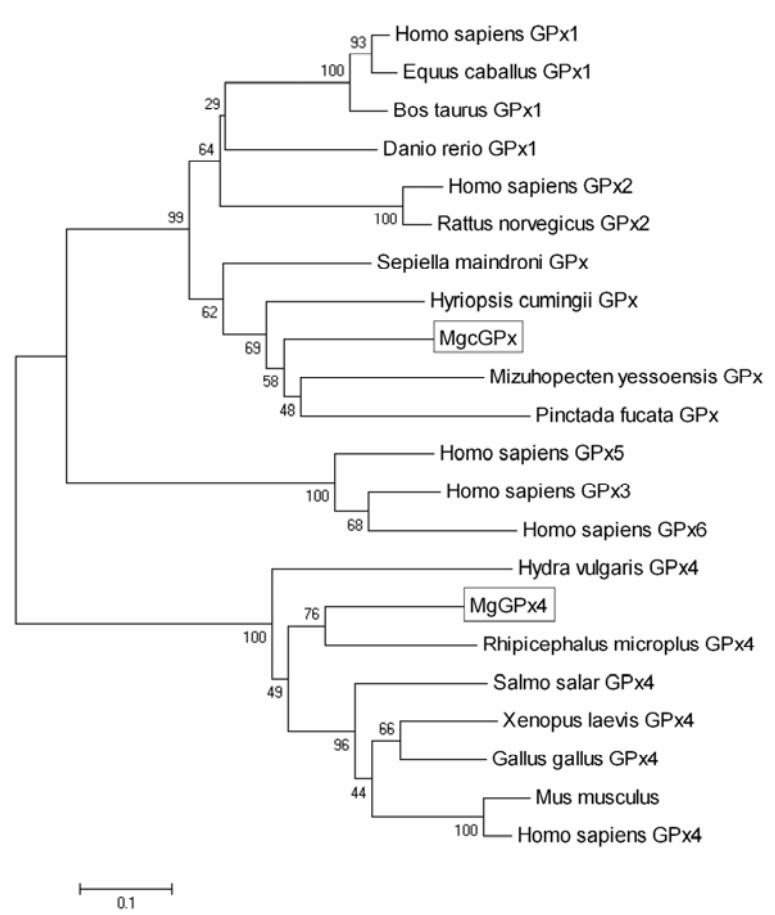

Fig. 3 Phylogenetic tree constructed by neighbour-joining method based on the sequences of GPxs from different animals. Numbers at the forks indicate the bootstrap values (in %) out of 1,000 replicates. The sequences used to construct phylogeny trees of GPxs are shown in Table 2.

Table 2 Sequences used for multiple sequence alignments and phylogenetic analysis

Gene	Species	Common name	Accession numbers
GPx1	*Homo sapiens*	human	NP_000572.2
GPx1	*Equus caballus*	horse	NP_001159951.1
GPx1	*Bos taurus*	cattle	NP_776501.1
GPx1	*Danio rerio*	zebra fish	NP_001007282.2
GPx2	*Homo sapiens*	human	NP_002074.2
GPx2	*Rattus norvegicus*	brown rat	NP_899653.2
GPx3	*Homo sapiens*	human	AAP50261.1
GPx4	*Rhipicephalus microplus*	tick	ABA62390.1
GPx4	*Hydra vulgaris*	hydroid	ABC25027.1
GPx4	*Xenopus laevis*	African clawed frog	NP_001165213.1
GPx4	*Salmo salar*	Atlantic salmon	ACH86324.1
GPx4	*Gallus gallus*	chicken	AAM18080.2
GPx4	*Mus musculus*	house mouse	BAA22780.1
GPx4	*Homo sapiens*	human	CAA50793.1
GPx4	*Mytilus galloprovincialis*	Mediterranean mussel	JQ866922
GPx5	*Homo sapiens*	human	CAA06463.1
GPx6	*Homo sapiens*	human	AAY68223.1
GPx	*Mizuhopecten yessoensis*	yesso scallop	ADQ92353.1
GPx	*Hyriopsis cumingii*	triangle shell mussel	ACY72387.1
GPx	*Sepiella maindroni*	cuttlefish	AEK48346.1
GPx	*Pinctada fucata*	pearl oyster	ADC35417.1
GPx	*Mytilus galloprovincialis*	Mediterranean mussel	HQ891311

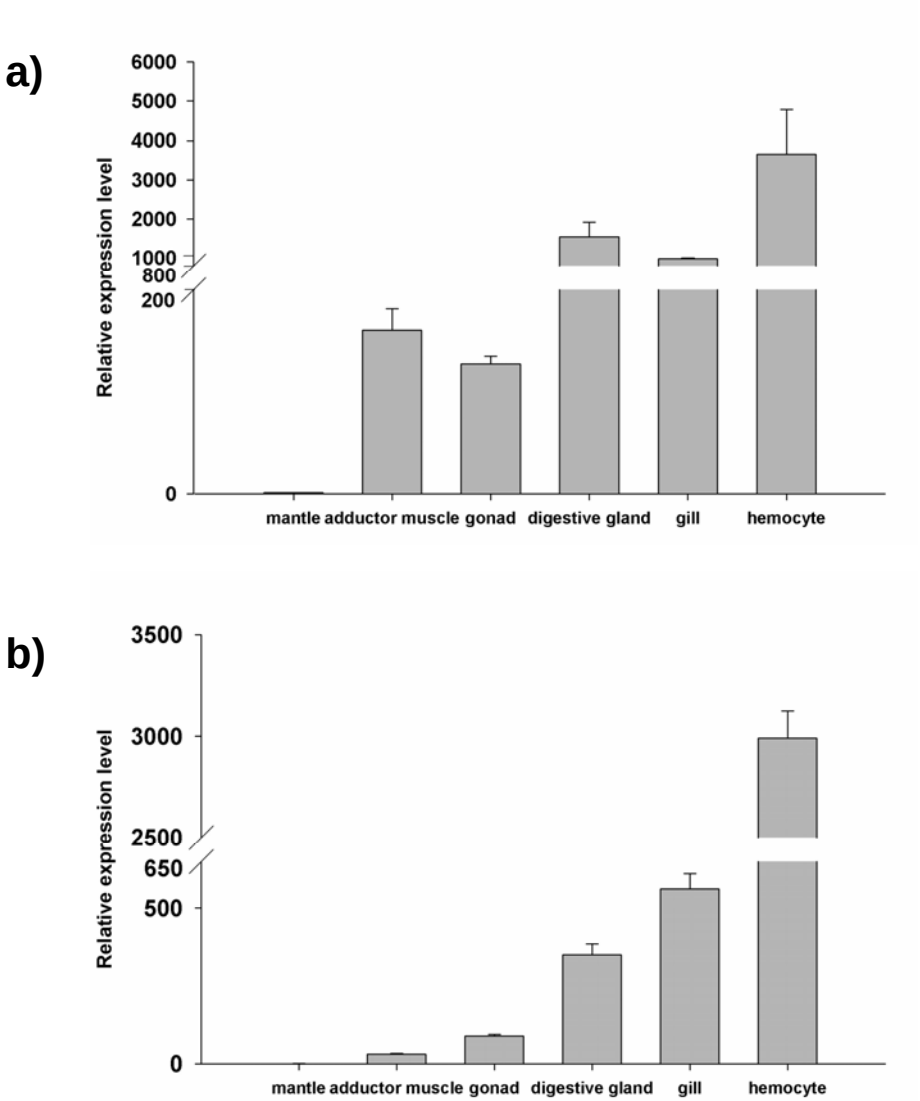

Fig. 4 Tissue-specific mRNA expression of MgcGPx (A) and MgGPx4 (B) determined by quantitative real-time PCR. Each bar represented mean ± SE (*n* = 4).

encoded a polypeptide of 199 amino acids with a predicted molecular weight of 22.97 kDa and a theoretical isoelectric point (pI) of 9.11. SMART program analysis revealed that MgcGPx contained a typical GSHPx domain ranging from Phe^{15} to Arg^{128}. The full-length cDNA of MgGPx4 encoded a polypeptide of 170 amino acids with a predicted molecular weight of 19.48 kDa and a theoretical pI of 8.62. SMART program analysis demonstrated that MgGPx4 also contained a GSHPx domain ranging from Ile^{14} to Lys^{123}. SignalP program analysis revealed that no putative signal peptide existed in the deduced amino acids of MgcGPx and MgGPx4. In addition, a 98 bp and a 93 bp SECIS element were identified in the 3' untranslated region (UTR) of MgcGPx and MgGPx4 cDNAs, respectively. Both the SECIS elements were predicted to form a stem-loop secondary structure and belonged to the form 2 SECIS element.

Multiple alignments and Phylogenetic analysis

Multiple alignments revealed that the characteristic Sec (U^{48}) residue, catalytically essential residues (Q^{82}, W^{160}), the typical GPx signature 2 motif ^{72}LGFPCNQF^{79} and the conserved active site motif ^{160}WNFEKF^{165} were well conserved in MgcGPx and other selected mollusk GPx sequences (Fig. 2A). Similarly, MgGPx4 also contained the characteristic residues (U^{46}, Q^{82} and W^{136}), the conserved ^{72}LGFPCNQF^{79} motif and ^{136}WNFTKF^{141} motif in its sequence (Fig. 2B).

To evaluate the molecular evolutionary relationship of MgcGPx and MgGPx4 against other GPxs, the amino acid sequences of 20 representative GPxs were selected to construct the phylogenetic tree (Fig. 3). The phylogenetic tree of GPxs included three major clades: the GPx1 and GPx2 (including GPxs from mollusks) clade; the GPx3, GPx5 and GPx6 clade; and the GPx4 clade.

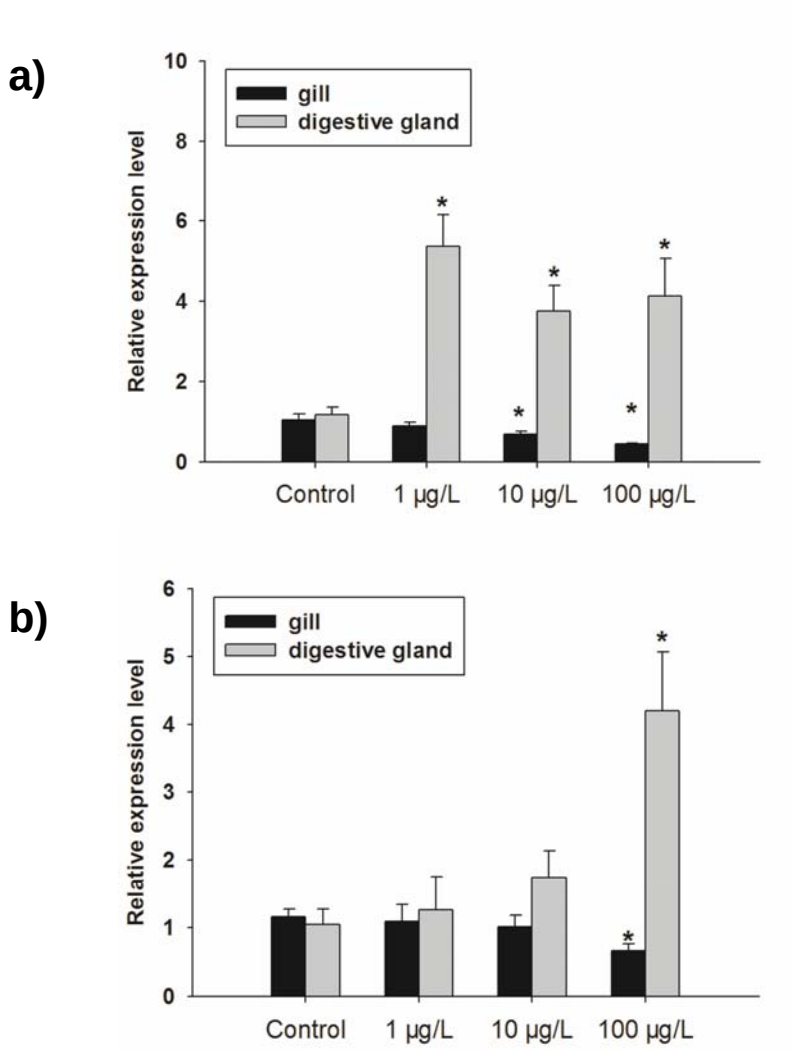

Fig. 5 Temporal mRNA expression profiles of MgcGPx (A) and MgGPx4 (B) mRNA in gill and digestive gland after Cd-exposure measured by quantitative real-time PCR. Each bar represented mean ± SE (n = 4). Significance across control was indicated with an asterisk at $p < 0.05$.

MgcGPx firstly clustered with GPxs from the yesso scallop *Mizuhopecten yessoensis* and pearl oyster *Pinctada fucata*, then formed a mollusc subgroup with GPxs from the triangle shell mussel *Hyriopsis cumingii* and the cuttlefish *Sepiella maindroni*, and it further grouped with typical GPx1s and GPx2s from vertebrates. In the GPx4 clade, MgGPx4 firstly grouped with GPx4s from the tick *Rhipicephalus microplus* and the hydroid *Hydra vulgaris* and then clustered with GPx4s from other vertebrates including fish, amphibian, bird and mammals. The phylogenetic analysis indicated that MgcGPx was derived from a common ancestor with most mollusc GPx family and MgGPx4 was a typical member of GPx4 family.

Tissue-specific distribution of MgcGPx and MgGPx4transcripts

GeNorm identified β-actin as the most stable gene with a V2/3 value 0.139 less than the proposed geNorm cutoff value of 0.15, which was then followed by ubiquitin C, α-tubulin, GAPDH, DDX and EF1. Therefore, β-actin was used as the internal control for gene expression normalization.

The tissue-specific distribution patterns of MgcGPx and MgGPx4 mRNA were investigated by qRT-PCR with β-actin as the internal control. For MgcGPx, MgGPx4 and β-actin genes, there was only one peak at the corresponding melting temperature in the dissociation curve analysis, indicating that the PCR was specifically amplified (data not shown). The transcripts of MgcGPx and MgGPx4 were detected in all the examined tissues, including digestive gland, gills, hemocytes, adductor muscle, mantle and gonad. The MgcGPx transcript was mainly detected in the tissue of hemcoytes, digestive gland and gills, moderately expressed in adductor muscle and gonad (Fig. 4A). A similar tissue-specific distribution pattern was also observed for MgGPx4 transcript with the highest expression level in hemocytes, moderate levels in gills and digestive gland, least level in mantle (Fig. 4B).

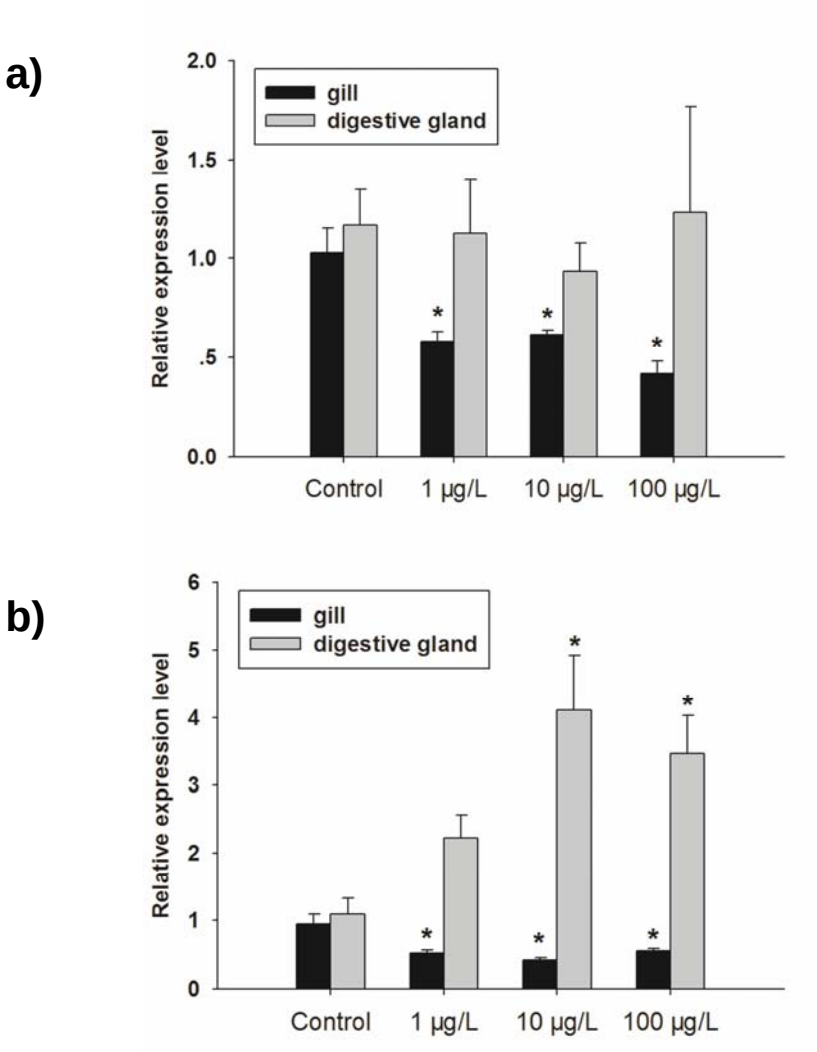

Fig. 6 Temporal mRNA expression profiles of MgcGPx (A) and MgGPx4 (B) mRNA in gill and digestive gland after As^V-exposure measured by quantitative real-time PCR. Each bar represented mean ± SE (n = 4). Significance across control was indicated with an asterisk at $p < 0.05$.

Transcriptional response of MgcGPx and MgGPx4 in gills and digestive gland exposed to Cd

After sub-chronic Cd exposure, the transcript level of MgcGPx was significantly down-regulated in gills at relative high concentrations (10 µg L^{-1} and 100 µg L^{-1}), whereas the transcriptional levels of MgcGPx increased significantly in digestive gland at all exposure concentrations compared to the control ($p < 0.05$) (Fig. 5A). However, a significantly higher transcriptional up-regulation of MgGPx4 in digestive gland was only observed in the mussels exposed to 100 µg L^{-1} Cd. In gills, the mRNA expression of MgGPx4 was also significantly inhibited at the highest concentration (100 µg L^{-1}) compared to that of the control ($p < 0.05$) (Fig. 5B).

Transcriptional response of MgcGPx and MgGPx4 in gills and digestive gland exposed to As^V

In gills, the transcription of MgcGPx was significantly inhibited at all exposure concentrations compared to the control. However, the mRNA expression level of MgcGPx in digestive gland had no significant change at all concentrations compared to the control ($p < 0.05$) (Fig. 6A). Similarly, the transcription level of MgGPx4 in gills significantly decreased at all concentration. The mRNA expression level of MgGPx4 was significantly higher in the digestive gland of mussels treated with higher concentrations (10 µg L^{-1} and 100 µg L^{-1}) compared to the control ($p < 0.05$) (Fig. 6B).

Alteration of total GPx activities in gills and digestive gland after Cd and As^V exposure

A significant decrease in GPx activity was observed in gills of the mussels exposed to 10 µg L^{-1} and 100 µg L^{-1} Cd compared to the control ($p < 0.05$). In digestive gland, the GPx activity increased significantly at all exposure concentrations (Fig. 7A). The modulation in transcript level of GPx coincided with the change in total GPx activity following Cd

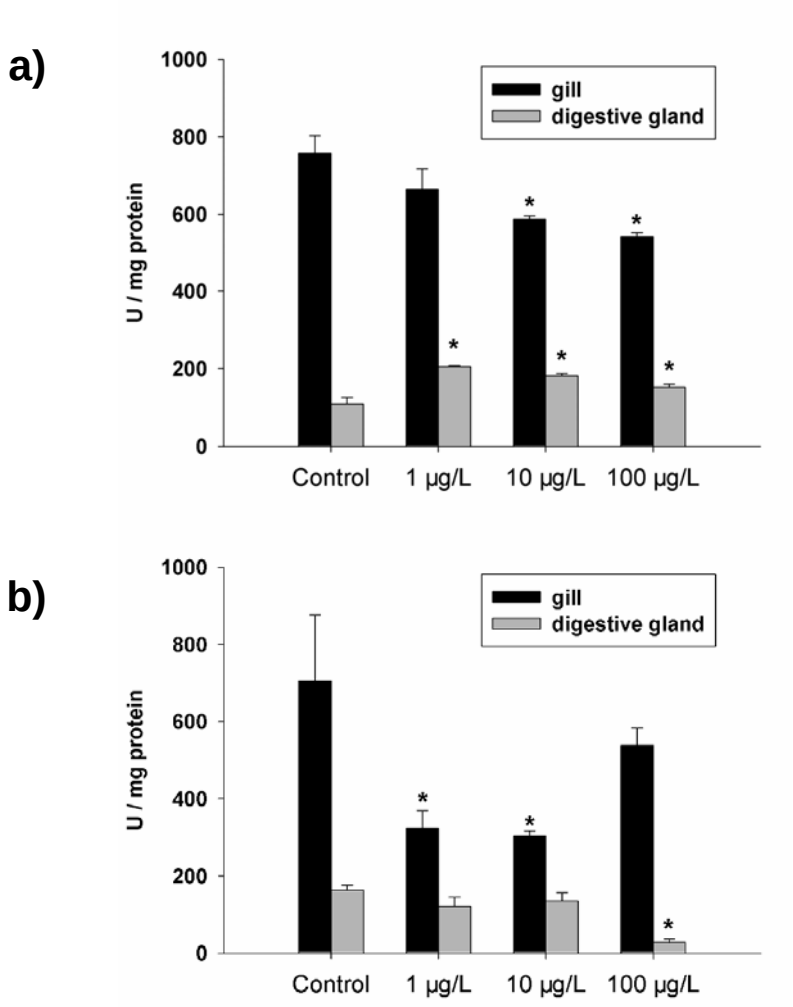

Fig. 7 Alteration of total GPx activities in gill and digestive gland after Cd (A) and As^{V} (B) exposure. Each bar represented mean ± SE (n = 4). Significance across control was indicated with an asterisk at $p < 0.05$.

exposure. After sub-chronic exposure, 1 µg L^{-1} and 10 µg L^{-1} As^{V} significantly inhibited GPx activity in gills ($p < 0.05$). Arsenate also inhibited GPx activity in digestive gland, and a statistically significant effect was observed in the 100 µg L^{-1} treated group (Fig. 7B). There was no obvious relation between gene expression and enzymatic activity of GPx following As^{V} exposure in this mussel.

Discussion

Glutathione peroxidase enzymes are involved in the elimination of ROS produced in physiological and pathological processes. Their differential expression as compared to normal expression pattern can indicate exposure to cellular stress and adverse cellular effects (Dash *et al*., 2006). Thus, GPxs have been proposed as biomarkers for the monitoring of physiological stress and toxicity induced by environmental toxicants in different aquatic organisms such as mollusks and fishes (Cossu *et al*., 1997; Sayeed *et al*., 2003; Almeida *et al*., 2004; Zhang *et al*., 2004; Valavanidis *et al*., 2006). In some field and laboratory studies, the GPx activities in *M. galloprovincialis* have been already examined to assess the toxic effects of contaminants in marine environment (Cravo *et al*., 2009; Maria *et al*., 2009; Chatziargyriou and Dailianis, 2010; Gomes *et al*., 2012; Nahrgang *et al*., 2013). However, no GPx genes have been characterized from this ecotoxicologically important species so far, although the mRNA expression of MgcGPx has been examined in mussels exposed to chemical mixtures (Giuliani *et al*., 2013).

In this study, typical mollusc GPx and GPx4 genes were cloned and characterized in *M. galloprovincialis*. Both amino acid sequences contained a selenocysteine encoded by a terminator codon TGA, suggesting that they belonged to the family of selenocysteine-containing proteins. Multiple sequence alignment showed that both sequences contained the conserved GPx signature motif 2 and active site motif, which strongly implied that they were new members of the

selenium-dependent GPx family. Phylogenetic analysis demonstrated that MgcGPx was similar to vertebrate GPx1s and GPx2s, indicating it might be a GPx1 or GPx2 isoform. Furthermore, the mollusc GPx branch was in the base of GPx1 and GPx2 branch, suggesting a parallel evolution among most mollusc GPxs and vertebrate GPx1/GPx2 (Fu *et al*., 2012).

Tissue-specific distribution patterns of MgcGPx and MgGPx4 transcripts indicated that both transcripts were mainly expressed in hemocytes, gills and digestive gland. In bivalves, ROS production had been shown to be involved in the immune defense and detoxification process of hemocytes (Gomez-Mendikute and Cajaraville, 2003). The comparably high expression levels of MgcGPx and MgGPx4 may be associated with the high oxidative stress occurring in hemocytes. The enrichment of MgcGPx and MgGPx4 transcripts in digestive gland was probably due to the fact that digestive gland was the major metabolic site for pollutants detoxification in molluscs (Livingstone, 1991). The high transcript level of GPx in digestive gland was also reported in scallops and clams (Mu *et al*., 2010; Wang *et al*., 2011a; Zhang *et al*., 2011). The gill is continuously exposed to environmental stress factors such as metallic ion, salinity, toxic substance and pathogen. Therefore, the transcripts of MgcGPx and MgGPx4 might be highly transcribed in this tissue. Similar results were also found in abalone, oyster and clam (De Zoysa *et al*., 2008; Jo *et al*., 2008; Zhang *et al*., 2011, 2012).

Cadmium and arsenic are prooxidant contaminants and exposure to them is often associated with antioxidant response in aquatic organisms (Winston and Di Giulio, 1991; Ventura-Lima *et al*., 2011). Recently, the change of GPx at transcriptional level has been used to evaluate the antioxidant response to contaminant stress in molluscs (Jo *et al*., 2008; Bigot *et al*., 2011, Zhang *et al*., 2011; Wang *et al*., 2012). Several studies have demonstrated that mollusc GPx activity can be modulated upon cadmium exposure (Chandran *et al*., 2005; Legeay *et al*., 2005; Wang *et al*., 2010; Wang *et al*., 2011b). However, the antioxidant response to arsenic exposure was less studied in molluscs (Chakraborty *et al*., 2010).

In this report, a dose-response study was carried out to elucidate gene expression profiles of MgcGPx and MgGPx4 upon contaminants exposure. In the gill tissues, the expression levels of both transcripts decreased in a dose-dependent manner after Cd exposure, although a significant decrease was only observed in relative high concentration. After As^V exposure, both transcripts of MgcGPx and MgGPx4 were significantly down-regulated at all concentrations in gills, indicating that both these genes responded sensitively to arsenic exposure even at the environmentally relevant concentration. However, another study reported that GPx transcript in gills of oyster *C. gigas* increased as the Cd concentration (10, 50 and 100 μg L^{-1}) and exposure time (11 day) increased. It was deduced that short term cadmium exposure might stimulate the mRNA expression of GPx to counteract the antioxidant stress in oyster (Jo *et al*., 2008). However, sub-chronic contaminant exposure might cause strong oxidative stress in gills which are usually constantly contacted with pollutants. The stress could dramatically lower the metabolic capacity and result in a decrease in mRNA expression of antioxidant enzymes (Zhang *et al*., 2004, 2011). Additionally, the tissue structure damage in gills of bivalve might result in the inhibition of GPx mRNA expression after As^V exposure (Chakraborty *et al*., 2010). It has also been reported that the mRNA expression of GPx4 was inhibited in fish olfactory tissue which was frequently contact with contaminant. In common carp, the mRNA expression of GPx4b was greatly down-regulated following Cd (10 mg L^{-1}) exposure for 21 d (Hermesz and Ferencz, 2009). In Coho salmon, both low (3.7 μg L^{-1}) and high (347 μg L^{-1}) level Cd exposures for 24 - 48 h significantly decreased GPx4a mRNA expression in olfactory (Wang *et al*., 2012). In addition, negative correlations were observed between the mRNA expression levels of MgcGPx and MgGPx4 in gills and the concentration of contaminant exposure, suggesting that the mRNA expression of these genes might be used as potential biomarkers for assessing the toxicity associated with contaminant exposures. Several previous studies also proposed the use of GPx4 transcript level in aquatic animals as biomarker for monitoring environmental pollution (Dash *et al*., 2006; Nair *et al*., 2012).

In digestive gland, Cd but not As^V exposure modulated the mRNA expression of MgcGPx at all concentrations, suggesting that MgcGPx was perhaps involved in the detoxification process of Cd in digestive gland. Similarly, the up-regulation of GPx mRNA expression had also been reported in digestive gland of *V. philippinarum* after Cd exposure (Zhang *et al*., 2011). However, few studies have investigated the possible role of bivalve GPx4 in the detoxification process. It was reported that GPx4 activity could be stimulated by Cd (200 μg L^{-1}) stress in digestive gland, indicating a protective role of GPx4 against lipid peroxidation in *P. perna* (Almeida *et al*., 2004). No information is available on the expression pattern of Gpx4 in response to pollutant in mollusc so far. In the present study, the transcription of MgGPx4 was up-regulated in mussels exposed to both Cd and As^V at the high concentration (100 μg L^{-1}). These results indicated that the transcript of MgGPx4 was modulated to protect the mussel against oxidative stress in the digestive glands of *M. galloprovincialis*. Similarly, the expression of GPx4 mRNA was also induced after Cd (49.2 mg L^{-1}) and As^V (24.0 mg L^{-1}) exposure for 6 h in hydra *H. vulgaris* (Dash *et al*., 2006). In the aquatic midge *C. riparius*, the expression of *GPx4* mRNA was found to be modulated upon exposure to different concentrations and time durations of Cd (Nair *et al*., 2012).

In this study, total GPx activity was also assayed to determine the correlation between the gene expression and enzymatic activity. It was found that the increase in transcript level of MgcGPx and MgGPx4 mainly coincided with the increase of total GPx activity following Cd exposure. However, the change of total GPx activity was different with the mRNA expression pattern of MgcGPx and MgGPx4 in digestive gland after As^V exposure. A different

sensitivity towards the inducers at the transcription level, mRNA processing, or transport and protein stability may lead to discrepancies between transcript level and enzyme activity (Okey, 1990). Moreover, MgcGPx and MgGPx4 contributed only partially to the total enzymatic activity of GPx in this mussel. The inhibition of GPx activity by arsenate might be ascribed to the fact that As^{V} can form complexes with GSH to facilitate the excretion and biotransformation processes of this element (Aposhian *et al*., 2004). Therefore, As^{V} might have adverse impact on the content of GSH in cells to affect the total GPx activity.

In summary, we have cloned two GPx genes (MgcGPx and MgGPx4) in *M. galloprovincialis*. Both the encoded GPx proteins had the conserved selenocysteine residue, catalytic sites and signature motifs. The constitutive transcripts of these genes were detected in six tissues examined. The modulation of MgcGPx and MgGPx4 transcript were involved in the antioxidant responses against pollutants challenge. This work will contribute to a better understanding of detoxification systems in marine bivalves. Moreover, the expression of MgcGPx and MgGPx4 mRNA can be considered as potential molecular biomarkers for assessing contaminant toxicity and aquatic environmental quality.

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