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Expression profiles of seven glutathione S-transferase (*GST*) genes from *Venerupis philippinarum* exposed to heavy metals and benzo[a]pyrene

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ABSTRACT

Glutathione S-transferases (GSTs) are phase II enzymes that facilitate the detoxification of xenobiotics, and also play important roles in antioxidant defense. In this study, we reported the cloning and molecular characteristics of seven genes of the GST family (*VpGSTS1*, *VpGSTS2*, *VpGSTS3*, *VpGSTO*, *VpGSTMi*, *VpGSTM* and *VpGSTR*) from *Venerupis philippinarum* together with mRNA tissue distribution patterns and temporal expression profiles in response to cadmium, copper and benzo[a]pyrene (B[a]P) exposures. The deduced amino acid sequences of *VpGSTs* showed high similarities to counterparts of other species that clustered into the same clades in the phylogenetic analysis. At basal levels of tissue expression, most *VpGSTs* were highly expressed in hepatopancreas compared with other tissues. All *VpGSTs* showed differential response profiles depending on the concentrations of various toxicants and exposure times. More notably, the expressions of *VpGSTS2* and *VpGSTS3* transcripts were significantly up-regulated in hepatopancreas from Cu and B[a]P-exposed animals, indicating that these two sigma *VpGSTs* were highly sensitive to Cu and B[a]P exposure. However, the expressions of *VpGSTM* and *VpGSTR* were significantly induced by Cu or B[a]P exposure, respectively. These findings suggested the role of *VpGSTS2*, *VpGSTS3*, *VpGSTM* and *VpGSTR* in defense against oxidative stress and highlighted their potential as biomarkers to Cu or B[a]P exposure.

1. Introduction

Aquatic organisms are frequently exposed to numerous environmental pollutants including polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Rainbow, 1995; Xue and Warshawsky, 2005; Wan et al., 2009). Heavy metals such as copper (Cu) and cadmium (Cd) can induce excessive production of reactive oxygen species (ROS) in cells, which cause oxidative modification of the major cellular macromolecules (Cecconi et al., 2002). Benzo[a]pyrene (B[a]P) is an extensively studied prototype of PAH and has been diffused into marine environment as an persistent organic pollutant, inducing cytotoxic, mutagenic and carcinogenic effects in various animal species (Gonzalez and Gelboin, 1994). In recent years, research on the gene expression of some detoxification and antioxidant enzymes has been recognized as a powerful tool to assess the ecotoxicological effects of certain chemicals (Woo et al., 2009; Won et al., 2011).

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a superfamily of multifunctional phase II enzymes primarily involved in detoxification of both endogenous and exogenous electrophiles. Generally, the detoxification mechanisms of GSTs lie in the conjugation of reduced glutathione with toxic compounds or their metabolites to increase the hydrophilicity and facilitate the excretion of toxicants (Ketterer et al., 1983). Moreover, GSTs have been found to play a critical role in mitigating oxidative stress in all life forms (Lee et al., 2008), and GST activities have been widely used as potential biomarkers for the monitoring of environmental pollution as well (Hoarau et al., 2002; Shailaja and D'Silva, 2003; Cunha et al., 2007).

GSTs have been found in all the prokaryotic and eukaryotic organisms investigated so far, in which they have three intracellular locations: in the cytosol, in mitochondria, and in microsomes (Hayes et al., 2005). Currently, at least 15 different classes of GSTs (alpha, beta, delta, epsilon, kappa, lambda, mu, omega, phi, pi, sigma, tau, theta, zeta, and rho) have been identified from numerous phylogenetically diverse organisms based on the differences in structural, catalytic and immunological characters (Hayes et al., 2005). In the previous studies, the mRNA expression of multiple specific *GSTs* had been investigated in several aquatic

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organisms after exposure to environmental pollutants (Boutet et al., 2004; Lee et al., 2008; Wan et al., 2008a; Li et al., 2009; Kim et al., 2010; Nail and Choi, 2011; Won et al., 2011). These studies provided the possibility to explore the correlation of specific *GST* responses with the detoxification of various toxicants, which were perhaps useful to evaluate the potential of different *GST* isoforms as molecular biomarkers to monitor marine environmental pollution. However, phylogenetically diverse organisms do not always respond similarly with regard to the transcriptional response of *GSTs*, therefore it is necessary to analyze the expression profiles of these genes across species.

Manila clam, *Venerupis philippinarum* is widely distributed and occurs at high densities around the heavily polluted Bohai Sea (Liang et al., 2004). It has been listed as a sentinel organism in "Mussel Watch Programs" launched in China. Our previous studies had demonstrated that this species exhibited distinct biochemical and genetic responses when exposed to heavy metals and B[a]P (Liu et al., 2011; Wang et al., 2011; Zhang et al., 2011a, 2011b), possessing the requisite features of a promising indicator species. Because GST isoenzymes were considered useful biomarkers in mollusk species (Boutet et al., 2004; Hoarau et al., 2006; Wan et al., 2008a, 2008b, 2009), investigation on the expression profiles of *VpGSTs* isoforms to specific environmental pollutants would be helpful to identify potential biomarkers for future environmental biomonitoring. To our knowledge, only one *GST-pi* gene was cloned and characterized from *V. philippinarum* (Xu et al., 2010). However, information about different classes of *VpGSTs* and their expression profiles to various toxicants exposure is currently unavailable. In this study, we reported the identification, characterization and distribution of seven *VpGSTs* from our ESTs database. To further identify promising *VpGST* isoforms as biomarkers, the expression patterns of seven *VpGSTs* were also investigated after exposure to three typical environmental contaminants in the Bohai Sea.

2. Materials and methods

2.1. Clams and treatments

The clams *V. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local aqufarm and acclimatized in aerated seawater (33 parts per thousand) at 25 °C for 10 days before exposures. During the acclimatization period, the clams were fed with *Chlorella vulgaris Beij*, and the water was totally exchanged daily. After the acclimatization, the clams were randomly divided into eight flat-bottomed rectangular tanks, each containing 50 individuals in 20 L seawater.

For the exposure experiment, fifty clams per tank were treated with different toxicants at following final concentrations: Cd (as $CdCl_2$, 10 and 40 μg/L), Cu (as $CuCl_2$, 10 and 40 μg/L) and B[a]P (5 and 50 μg/L dissolved in DMSO). The concentration of heavy metals and B[a]P have been previously reported in some heavily polluted sites of the Bohai Sea and the coastal waters of southeast China (Zhang, 2001; Zhang et al., 2004). The clams cultured in the normal filtered seawater (FSW) and FSW containing 0.002% DMSO (v/v) were used as the blank and control groups, respectively. During exposure time, clams were also fed with *Chlorella vulgaris Beij* daily in FSW for 2 h, and then transferred into renewed toxicant-laden seawater daily. The hepatopancreas of four individuals from each treatment were separately sampled after exposure for 24, 48 and 96 h respectively. After collection, the samples were flash-frozen in liquid nitrogen immediately and stored at −80 °C for subsequent total RNA extraction. Meanwhile, gills, hepatopancreas, adductor muscles, mantles and haemocytes from four untreated clams were also collected to determine the tissue distribution of *VpGSTs*.

2.2. Cloning the full-length cDNA of VpGSTs

Partial sequences of seven *GST* genes were obtained from a cDNA library of *V. philippinarum*. To determine the full-length cDNA of *VpGSTs* genes, twenty-eight specific primers (Table S1) were designed based on the above fragments. The nested PCR strategy was applied to the 3' and 5' RACE. The RNA extraction, cDNA synthesis, PCR amplification and PCR product sequencing were performed according to previously described (Wang et al., 2011).

The obtained *GST* sequences were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The percentages of similarity and identity of VpGSTs with GST proteins from other organisms were calculated by the Identity and Similarity Analysis program (http://www.biosoft.net/sms/index.html). To investigate the phylogenetic relationship of VpGSTs with GST proteins from other species, a phylogenetic tree was constructed with MrBayes version 3.2.0 (Ronquist and Huelsenbeck, 2003). The tree was built using the 'mixed-model' appraoch; 250,000 generations with chains sampled every 1000 generations and a burnin of 50,000 were used; and the WAG model was selected as the best fitting substitution model by MrBayes. The tree was visualized using TreeView. All GST protein sequences were retrieved by searching the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. VpGSTs mRNA expression in different tissues and the response to Cd, Cu and B[a]P exposure

Tissue distribution of *VpGSTs* and the response to Cd, Cu and B[a]P challenge were measured by quantitative real-time RT-PCR in Applied Biosystem 7500 fast Real-time PCR System. Details of the primers used for RT-PCR were given in the supplemental Table S1. β-Actin in *V. philippinarum* was used as a reference gene as previously described (Zhang et al., 2011a), which was frequently used in gene quantitative analysis after exposure to various chemicals (Doyen et al., 2006; Gao et al., 2007; Park et al., 2009). In a 96-well plate, each sample was run in triplicate along with the internal control. The PCR amplification was carried out in a total volume of 50 μL, containing 25 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 20 μL of the diluted cDNA, 0.5 μL of each of primers (10 μmol/L), and 4 μL of DEPC-treated water. The thermal profile for RT-PCR was 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 31 s. At the end of each PCR reaction, a dissociation curve analysis of amplification products was performed to confirm that only one PCR product was amplified and detected. Moreover, each real-time RT-PCR product was purified and sequenced to verify the PCR specificity. After amplification, data acquisition and analysis were performed using the Sequence Detection Software 2.3 (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was chosen as the calculation method. All datasets were given in terms of relative mRNA expression as means ± standard deviation. One-way analysis of variance (one-way ANOVA) was performed on all data using SPSS 13.0 statistical software, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Identification and classification of VpGSTs genes

Seven different *GST* transcripts were identified from the *V. philippinarum* EST database and the full-length cDNAs were cloned by RACE approaches. Based on the deduced amino acid identities and phylogenetic analysis with other *GSTs*, the *V. philippinarum GSTs* were classified into five different classes, including three in Sigma (*VpGSTS1*, *VpGSTS2*, *VpGSTS3*) and one each in Omega (*VpGSTO*), Mu

(*VpGSTM*), Rho (*VpGSTR*) and the microsomal GST isoenzyme (*VpGSTMi*). The cDNA and deduced amino acid sequences of the *VpGSTs* described in this study were deposited in GenBank under the following accession numbers: *VpGSTS1* (JN388948), *VpGSTS2* (JN388949), *VpGSTS3* (JN388950), *VpGSTO* (JN388951), *VpGSTMi* (JN388952), *VpGSTM* (JN388953) and *VpGSTR* (JN388954). *VpGSTMi* consisted of an open reading frame (ORF) of 450 bp encoding a protein of 149 residues with the predicted molecular mass of 16.61 kDa. VpGSTS, VpGSTS2, VpGSTS3, VpGSTO, VpGSTM and VpGSTR consisted of 203, 217, 210, 240, 217, and 225 amino acids, respectively. Among the cytosolic GSTs, VpGSTO had the highest molecular mass (27.5 kDa) and VpGSTS1 had the lowest (23.3 kDa), which was consistent with most mammalian GSTs with a molecular mass ranging from 23 to 28 kDa as homodimers or heterodimers (Blanchette and Singh, 2002). The putative VpGSTs proteins were calculated to have a theoretical pI of 5.79 to 8.91. The seven different VpGSTs were annotated using BlastX and each showed high similarities with that from other species including fish, mollusks and certain invertebrates (Table 1). The percentages of deduced amino acid identities were 43.3–45.2% among the three sigma GSTs, but only 11.8 to ≤38.5% among different classes of the VpGSTs.

The assignment of the seven VpGSTs to the sigma, omega, mu, rho and microsomal GST isoenzymes was clearly supported by our phylogenetic analysis of all the seven VpGSTs along with those from other species, including both vertebrates and invertebrates (Fig. 1). The phylogenetic tree showed that VpGSTs were separated into five groups, and each class formed their own clades. *V. philippinarum* GSTs were placed near mollusks and insects in the case of cytosolic GST.

3.2. Tissue distribution of VpGSTs mRNA

Tissue-specific expression patterns of the seven *V. philippinarum GST* genes were analyzed in each of five different tissues, including gills, hepatopancreas, adductor muscles, mantles, and haemocytes. Our results indicated that the seven *GST* genes were expressed in all tissues examined, although there were some noticeable variations in expression levels among different tissues (Fig. 2). As shown in Fig. 2, the highest levels of *VpGSTS1*, *VpGSTO*, *VpGSTMi*, *VpGSTM*, and *VpGSTR* mRNA expression were found in hepatopancreas (Fig. 2A,D, E,F and G). In contrast, *VpGSTS2* and *VpGSTS3* were found to be most abundantly expressed in the gills and haemocytes, respectively (Fig. 2B and C). In this study, the transcript levels of the seven *GST* isoforms were variably expressed in different tissues, and the abundance of different *GST* isoforms was also variable within a single tissue (Fig. 3). Specifically, the transcripts of *VpGSTS1* and *VpGSTM* were dominantly expressed in hepatopancreas, while *VpGSTS2* was expressed at a relatively low level in normal clam hepatopancreas.

3.3. Effect of Cd on VpGSTs expression

Messenger RNA expression of *GSTs* in *V. philippinarum* hepatopancreas showed differential expression patterns during Cd exposure at different concentrations and time intervals (Fig. 4). No significant change was observed in the mRNA expression of *VpGSTS1*, *VpGSTO*, *VpGSTMi* and *VpGSTM* at the tested concentrations throughout the time course (only in a few cases did these transcripts increase less than 2 fold with $P>0.05$) (Fig. 4A,D–F), while the expression levels of *VpGSTS2*, *VpGSTS3* and *VpGSTR* were significantly up- or down-regulated with exposures to Cd at different concentrations and time intervals. Among the different sigma class *VpGSTs*, *VpGSTS3* transcript was significantly up-regulated (less than 2 fold) after treated with 40 μg/L Cd for 24 h (Fig. 4C). However, the expression level of *VpGSTS2* dropped obviously to 0.07-fold ($P<0.05$) of control group at 48 h with 10 μg/L Cd exposure. When the concentration increased to 40 μg/L, the expression of *VpGSTS2* mRNA was significantly depressed after exposure for 24 (0.35-fold), 48 (0.17-fold) and 96 h (0.04-fold) compared with that of control group (Fig. 4B). As concerned to *VpGSTR*, the mRNA expression level was significantly depressed (0.46-fold) by 40 μg/L Cd after exposure for 96 h (Fig. 4G).

3.4. Effect of Cu on VpGSTs expression

In this study, Cu exposure affected the mRNA expression of several *VpGST* isoforms differentially (Fig. 5). No significant difference of *VpGSTR* expression level was observed from Cu-exposed samples at all time points (Fig. 5G). The expression levels of other *VpGST* genes were significantly up or down-regulated at different concentrations and time intervals. *VpGSTS1*, *VpGSTS3* and *VpGSTM* showed somewhat similar expression patterns, with significant increases (2.5–4.8 fold) after exposure to 10 and 40 μg/L of Cu at different exposure time intervals (Fig. 5A,C and F). However, the expression of *VpGSTS2* was significantly higher than those of the other sigma *VpGSTs* with an 11 fold increase after exposure for 96 h with 40 μg/L Cu (Fig. 5B). A significant increase in mRNA expression (2.4 fold) was also observed for *VpGSTO* with exposure to 10 μg/L of Cu for 96 h, whereas exposure to 40 μg/L Cu significantly reduced *VpGSTO* mRNA levels (0.13 fold at both 48 and 96 h) (Fig. 5D). With respect to *VpGSTMi*, the expression level decreased significantly (0.25 fold) at 24 h with 10 μg/L Cu exposure. When the concentration increased to 40 μg/L, the expression level of *VpGSTMi* was negatively affected with approximate decreases of 0.1 fold at 24 h, 0.07 fold at 48 h, and 0.17 fold at 96 h (Fig. 5E).

3.5. Effect of B[a]P on VpGSTs expression

The mRNA expression of *VpGSTs* in B[a]P-exposed *V. philippinarum* showed significantly different expression profiles (Fig. 6). For example, the expression of *VpGSTS1* dropped to 0.56 fold of the control group after exposed for 24 h with 5 μg/L of B[a]P. As time progressed, the expression of *VpGSTS1* recovered to the original level at 48 h. The *VpGSTS1* level at a high B[a]P (50 μg/L) concentration showed relatively stable expression with an exception of significant decrease (0.42 fold) at 96 h (Fig. 6A). In the case of *VpGSTO* and *VpGSTMi*, the transcriptional repression were also observed in 50 μg/L B[a]P treated group at 24 h. However, no significant changes in transcription were observed after treatment with 5 μg/L of B[a]P (Fig. 6D,E).

Table 1
BlastX result for seven different *GST* classes in the clam *V. philippinarum*.

Name	Closest Species	AC*	Gene	Identities
VpGSTS1	*Branchiostoma floridae*	XP_002610389	*GST-Sigma*	92/204 (45%)
VpGSTS2	*Venerupis philippinarum*	ADI44317	*GST-Sigma*	98/209 (47%)
VpGSTS3	*Venerupis philippinarum*	ADI44317	*GST-Sigma*	77/203 (38%)
VpGSTO	*Ictalurus punctatus*	ABD77536	*GST-Omega*	82/165 (50%)
VpGSTM	*Thais clavigera*	ACD13785	*GST-Mu*	123/216 (63%)
VpGSTR	*Laternula elliptica*	ACM44933	*GST-Rho*	1115/225 (51%)
VpGSTMi	*Dermacentor variabilis*	ACF35503	*GST-microsomal*	62/112 (55%)

* AC indicates Genbank accession number of closest species.

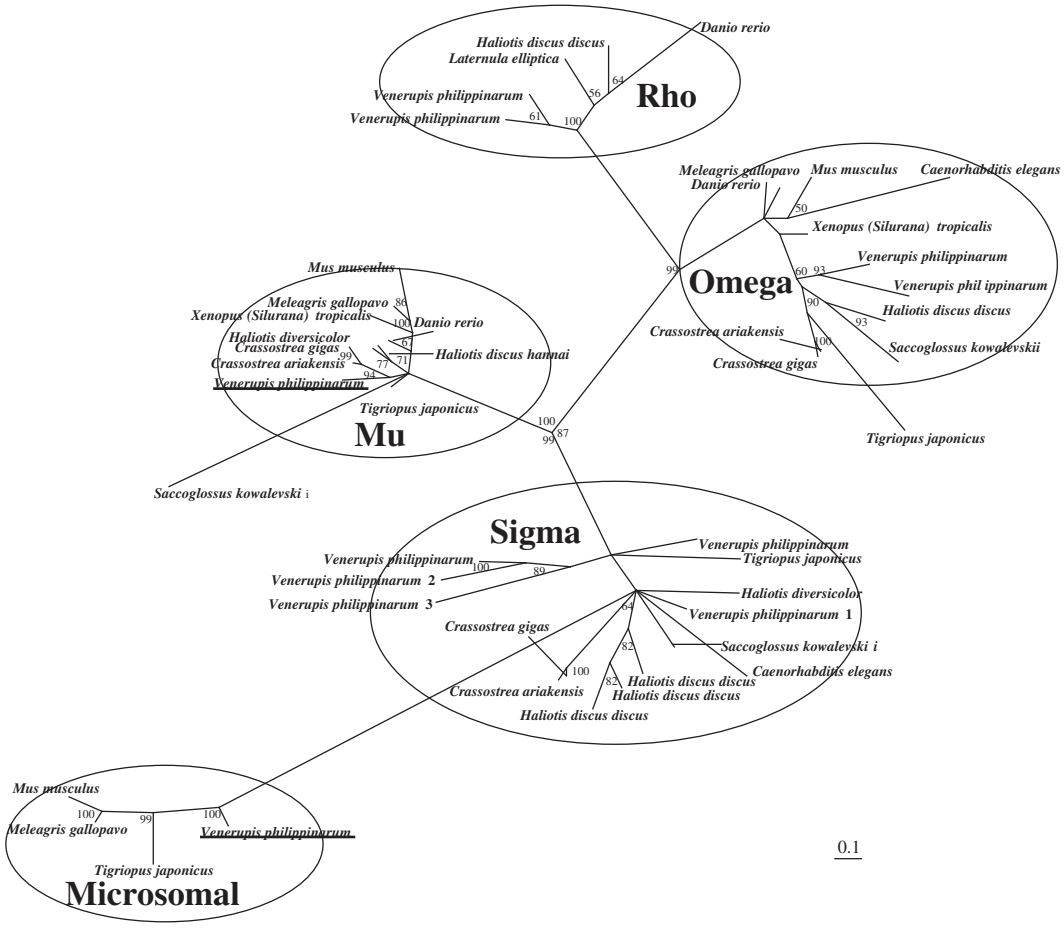


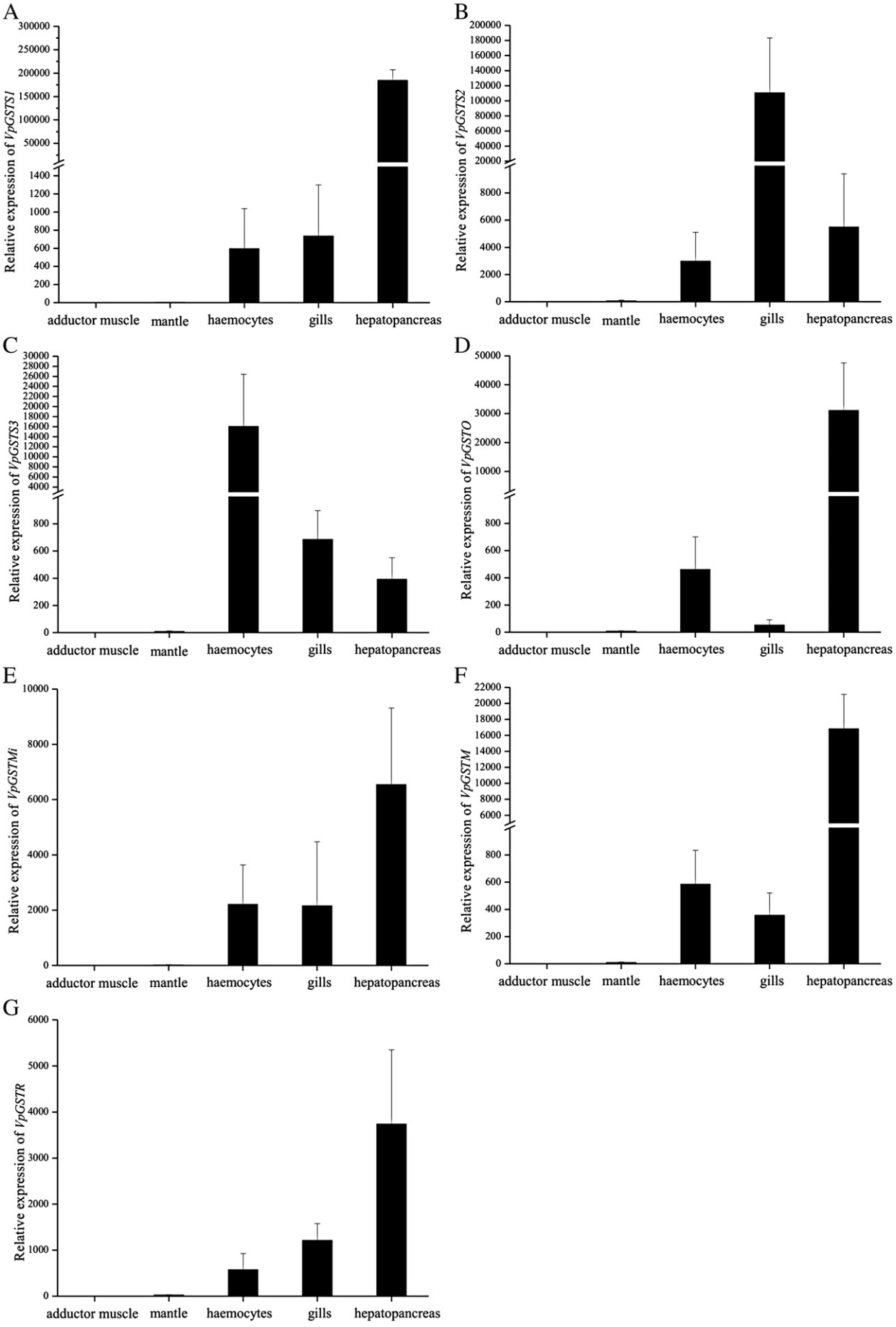
Fig. 1. Phylogenic tree of GSTs from *V. philippinarum* and other organisms. The values shown at the nodes of the branches are the confidence levels from 1000 replicate bootstrap samplings. The scale bar indicates the evolutionary distance between the groups. The amino acids sequence used in phylogenetic analysis are their Genbank accession numbers were as follows: GST-Sigma: *Venerupis philippinarum* (ACU83216, ADI44317), *Tigriopus japonicus* (AAY89317), *Haliotis diversicolor* (ABV01122), *Saccoglossus kowalevskii* (XP_002731487), *Caenorhabditis elegans* (NP_506983), *Haliotis discus discus* (ABO26602, ABO26603, ABO26604), *Crassostrea ariakensis* (ACJ06744), *Crassostrea gigas* (CAE11863); GST-Omega: *V. philippinarum* (ADI44318), *Danio rerio* (NP_001002621), *Meleagris gallopavo* (XP_003208190), *Mus musculus* (NP_034492), *Caenorhabditis elegans* (NP_498728), *Xenopus* (*Silurana*) *tropicalis* (CAJ81793); *Haliotis discus discus* (ABO26600), *S. kowalevskii* (XP_002738513), *Tigriopus japonicus* (ACE81246), *Crassostrea gigas* (CAD89618), *Crassostrea ariakensis* (ACJ06747); GST Mu: *T. japonicus* (ACE81254), *Saccoglossus kowalevskii* (XP_002742308), *Crassostrea ariakensis* (ACJ06748), *Crassostrea gigas* (CAD90167), *H. discus hannai* (ACE79172), *Haliotis diversicolor* (ABY87352), *Xenopus (Silurana) tropicalis* (NP_001004964), *M. gallopavo* (AEI70736), *M. musculus* (CAA04061); GST-Rho: *V. philippinarum* (ACU83218), *Laternula elliptica* (ACM44933), *H. discus discus* (ABO26605), *D. rerio* (BAF47196); GST Microsomal: *T. japonicus* (ACE81249), *M. gallopavo* (XP_003208606), *M. musculus* (NP_079845).

The expression level of *VpGSTS2* increased up to 4.5 fold after 48 h exposure to 5 μg/L of B[a]P, but a downward trend was noticed thereafter. The maximum increase of *VpGSTS2* expression (6.5 fold) was observed at 24 h after exposed to 50 μg/L B[a]P (Fig. 6B). *VpGSTR* also showed a higher level of expression compared to other up-regulated *GSTs*, with the highest expression level (6.5 fold) observed after 50 μg/L B[a]P exposure for 96 h (Fig. 6G). *VpGSTS3* displayed less notable increases (1.7–3.8 fold) after 24 and 48 h exposure to different concentrations of B[a]P, and then reduced to the control level at 96 h (Fig. 6C). As concerned to *VpGSTM*, the mRNA expression level was slightly induced with a 2.14-fold increase ($P<0.05$) after treated with 5 μg/L B[a]P for 48 h. However, exposure to higher doses of B[a]P (50 μg/L) resulted in less increases in the transcription level (Fig. 6F). Although the seven *VpGSTs* expressions in the blank and control groups were slightly fluctuant at different time points, no significant difference was found throughout the experiment (data not shown).

4. Discussion

The molecular biological approach in studying potential biomarker has been widely used as a reliable means to conduct environmental monitoring studies (Hoarau et al., 2006; Rhee et al., 2007). Owing to the sensitive response of mRNA expression to diverse forms of stressors, changes in mRNA expression of cytochrome P450, glutathione S-transferases, superoxide dismutase, heat shock proteins, metallothioneins and vitellogenin have been frequently used for the diagnosis of environmental contamination (Hoarau et al., 2006; Sarkar et al., 2006; Gao et al., 2007). Since GST play important roles in detoxification pathways and in the oxidative stress response, GSTs have been investigated widely in ecotoxicology and marine pollution assessment (Boutet et al., 2004; Hoarau et al., 2006; Rhee et al., 2007; Lee et al., 2008; Wan et al., 2008a, 2008b, 2009; Kim et al., 2010; Nail and Choi, 2011; Won et al., 2011). However, as a biomonitor in marine environmental monitoring, the information about

Fig. 2. Distribution of (A) *VpGSTS1* (B) *VpGSTS2* (C) *VpGSTS3* (D) *VpGSTO* (E) *VpGSTMi* (F) *VpGSTM* and (G) *VpGSTR* mRNA transcripts in different tissues. *VpGSTs* transcript level in haemocytes, gills, mantles, and hepatopancreas was normalized to that of adductor muscles and shown as mean ± standard deviation ($n=4$).


A
Relative expression of VpGSTS1
adductor muscle
mantle
haemocytes
gills
hepatopancreas
B
Relative expression of VpGSTS2
C
Relative expression of VpGSTS3
D
Relative expression of VpGSTO
E
Relative expression of VpGSTMi
F
Relative expression of VpGSTM
G
Relative expression of VpGSTR

various classes of *V. philippinarum GSTs* and their spatial and temporal expression profiles to heavy metals and B[a]P exposure is currently unavailable. In the following subsections, the transcriptional changes are discussed in regards to the tissue distribution, the susceptibility of each *VpGST*, as well as the biological effects of each chemical.

4.1. Tissue distribution of VpGSTs mRNA

In the glutathione S-tranferase family, there are at least 15 subclass members exhibiting different primary structures, enzyme properties, and physiological functions (Hayes et al., 2005). According to the functional differences, *GST* isoforms would express differentially in various tissues. Presently, investigations on the tissue distribution of *GSTs* in marine organisms have revealed that *GSTs* are generally abundantly expressed in the gills, hepatopancreas, gonad and mantle (Lee et al., 2005; Wan et al., 2008a; Ren et al., 2009a; Kim et al., 2010; Xu et al., 2010). Doi et al. (2004) and Lee et al. (2006) suggested that differences in tissue distribution of *GST* were associated with differential susceptibility to antioxidant damage. In the present study, *VpGSTS1*, *VpGSTO*, *VpGSTMi*, *VpGSTM* and *VpGSTR* displayed tissue-specific expression in hepatopancreas, consistent with the viewpoint that hepatopancreas was the major organ for detoxification of xenobiotics (Doi et al., 2004). In addition, *VpGSTS2* transcript was found to be predominantly expressed in gills, whereas *VpGSTS3* exhibited high expression levels in haemocytes. These results were somewhat different from many previous studies, suggesting that certain *GST* members might have some other functions, such as immune modulation observed in abalones and shrimps (Ren et al., 2009a, 2009b). More notably, *VpGSTS2* was expressed at a relatively low level in hepatopancreas of control clams (Fig. 3), but was remarkably up-regulated after the exposure with Cu and B[a]P (Fig. 5B, Fig. 6B). Previous studies had suggested that low constitutively expressed *GST* perhaps performed a crucial role in the detoxification process, whereas high constitutively expressed *GSTs* might involve in protecting the cell against endogenous oxidative stress (Lee et al., 2006; Liao et al., 2006; Ren et al., 2009b). It could be speculated that *VpGSTS2* perhaps played a vital role in the detoxification process and could be used as potential biomarker for Cu and B[a]P pollution.

4.2. Response of each VpGST to chemical exposures

The expression profile of seven *VpGST* genes was studied with an objective to identify the promising *GST* biomarkers. Among the three sigma class *VpGSTs*, *VpGSTS2* were significantly down-regulated to Cd exposure while *VpGSTS3* showed a significant up-regulation after treated with 40 μg/L Cd for 24 h. *VpGSTS1*, *VpGSTS2* and *VpGSTS3* were all strongly induced by Cu exposure with the highest expression level for *VpGSTS2* (11-fold). With regards to B[a]P exposure, the expression of *VpGSTS1* showed a dramatic decrease, while *VpGSTS2* and *VpGSTS3* displayed notable increases with larger amplitude in *VpGSTS2* (6.5 fold). It has been suggested that sigma class GSTs participate in cellular xenobiotic defenses and antioxidant systems in various organisms (Singh et al., 2001; Lee et al., 2008; Wan et al., 2008a; Nail and Choi, 2011; Won et al., 2011). For example, *Tigriopus japonicus GST* class sigma (*GST-S*) was observed to be more consistent than other types of *GSTs* both in concentration- and time-dependent studies when exposed to hydrogen peroxide and heavy metals (Lee et al., 2008). A sigma *GST* from disk abalone (*HdGSTS1*) also exhibited a considerable inducibility by organic contaminants (Wan et al., 2008a). Recent studies showed that sigma *GSTs* from *Chironomus riparius* and *Perinereis nuntia* displayed great potential as a biomarker to cadmium and silver nanoparticles exposure (Nail and Choi, 2011; Won et al., 2011). The results in the present study were positively consistent with previous reports, clearly demonstrating that some sigma class *VpGSTs* were more sensitive than other *GSTs* for heavy metals and B[a]P exposure.

GST omega isoenzymes were reported to take part in the cellular response to oxidative stress (Board et al., 2000). As reported, the expressions of omega *GSTs* in *T. japonicus, Takifugu obscurus*, *P. nuntia*, and *Haliotis discus discus* were significantly induced by Cd or Cu exposure (Lee et al., 2008; Wan et al., 2009; Kim et al., 2010; Won et al., 2011). In this work, *VpGSTO* showed a distinct response that was dramatically depressed by 40 μg/L Cu and 50 μg/L B[a]P. In the case of *VpGSTMi*, a similar pattern of transcriptional repression was observed by Cu and B[a]P exposure. However, the microsomal GST activities in rats were increased during the exposure of phenol (Wallin and Morgenstern, 1990) and hydrogen peroxide (Aniya and Anders, 2002). Microsomal GST was ubiquitously expressed and predominantly located in the endoplasmic reticulum and outer mitochondrial membrane, and was vital for the protection of organisms against agents that were known to induce lipid peroxidation (Johansson et al., 2010). It has been reported that many organisms over expressed GSTs on exposure to chemicals (Hayes et al., 2005). In *T. obscurus*, *GST-Mu* was highly expressed in liver compared with other tissues, and showed the highest expression among the up-regulated *GSTs* (Kim et al., 2010). From our studies, *VpGSTM* also showed significant increases (2–4.8 fold) after exposure to Cu with different exposure times, suggesting its protective role in the defense against oxidative stress. Meanwhile, the expression level of *VpGSTR* increased up to 6.5 fold after 96 h of exposure to 50 μg/L of B[a]P. Similar expression pattern was also observed in gills and digestive gland of Antarctic bivalve after exposure to 10 μg/L Aroclor 1254 (Park et al., 2009). However, the transcriptions of *GST rho* from goldfish and common carp were inhibited by cyanobacterial crude extract and microcystin (Fu and Xie, 2006; Hao et al., 2008).

Taken together, our results showed that Cd, Cu and B[a]P exposures caused differential expressions of the seven *VpGSTs* transcripts. The selective up- or down-regulation of *VpGST* gene expression highlighted the fact that these isoenzymes probably played divergent physiological roles during the detoxification of various pollutants in *V. philippinarum*. Among these *VpGSTs*, *VpGSTS2*, *VpGSTS3*, *VpGSTM* and *VpGSTR* were highly expressed in response to Cu or B[a]P exposure, demonstrating their great potential as molecular biomarkers for environmental biomonitoring studies.

4.3. Biological effects of each chemical

In this study, we selected three representative chemicals (Cd, Cu and B[a]P) commonly found in the marine environment to investigate the effect of each chemical on the transcription of different family of *VpGSTs*. It has been well acknowledged that Cd and Cu could induce the production of excessive ROS, resulting in oxidative stress thus affecting the antioxidant enzyme system (Cecconi et al., 2002). Previous studies have reported that exposure to Cu and Cd induces the expression of *GST* transcripts in many aquatic organisms (Yoshinaga et al., 2007; Lee et al., 2008; Kim et al., 2010). In this work, the expression of *VpGSTS2* and *VpGSTR* were significantly depressed by Cd, while the transcriptional induction of *VpGSTS2* and *VpGSTM*, as well as the

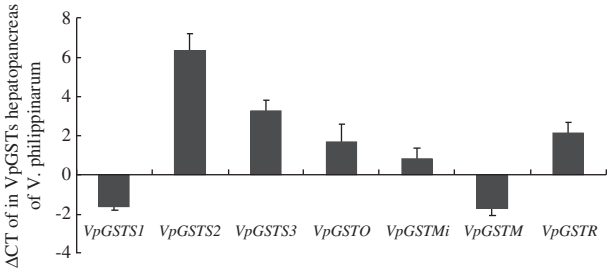


Fig. 3. Quantification of abundance of different *VpGST* isoforms in hepatopancreas of untreated clams. The abundance were calculated relative to actin expression and shown as mean ± standard deviation ($n = 4$).

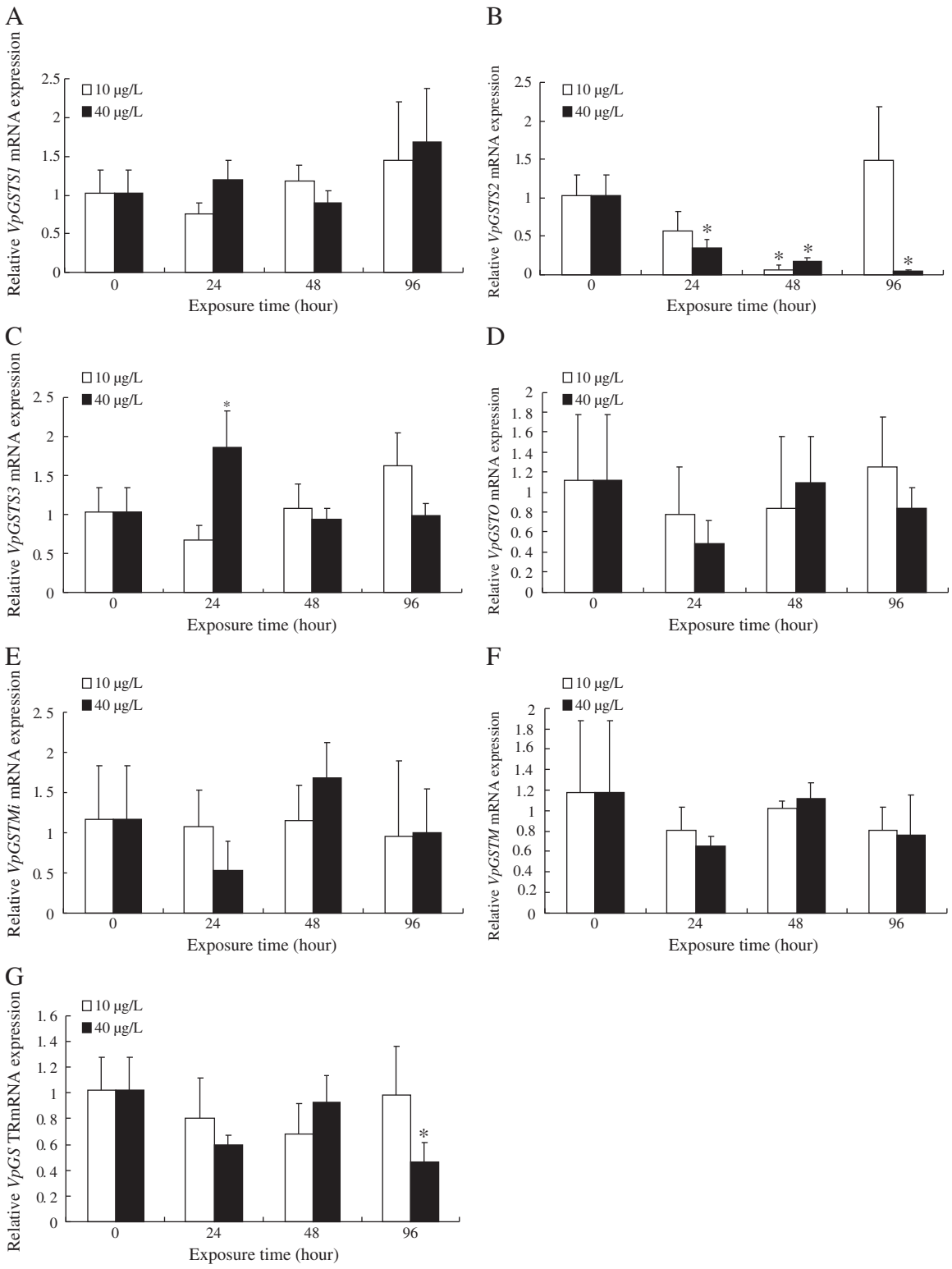


Fig. 4. Expression profiles of mRNA of (A) *VpGSTS1* (B) *VpGSTS2* (C) *VpGSTS3* (D) *VpGSTO* (E) *VpGSTMi* (F) *VpGSTM* and (G) *VpGSTR* after treatment with 10 and 40 μg/L Cd for 24, 48 and 96 h. *VpGSTs* mRNA expression level values were calculated relative to actin expression and shown as mean ± standard deviation ($n = 4$). Astericks indicate significant difference ($P<0.05$) as compared to the control.

repression of *VpGSTO* and expression of *VpGSTMi* were found in *V. philippinarum* exposed to Cu contamination. It has been suggested that chemical exposures might influence transcription regulatory factors that bind to the promoter region of some *GST* genes and mediate the transcription of specific GST isoenzymes (Fu and Xie, 2006; Kim et al., 2010). Moreover, the significant down-regulation of *GST* might be caused by the accumulation of GSH conjugation with chemicals, which was an inhibitor of *GST* expression (Srivastava et al., 1998, 1999). Future studies should explore the repression of *VpGSTS2* and *VpGSTR* gene expression after exposure to Cd, as well as the reduced

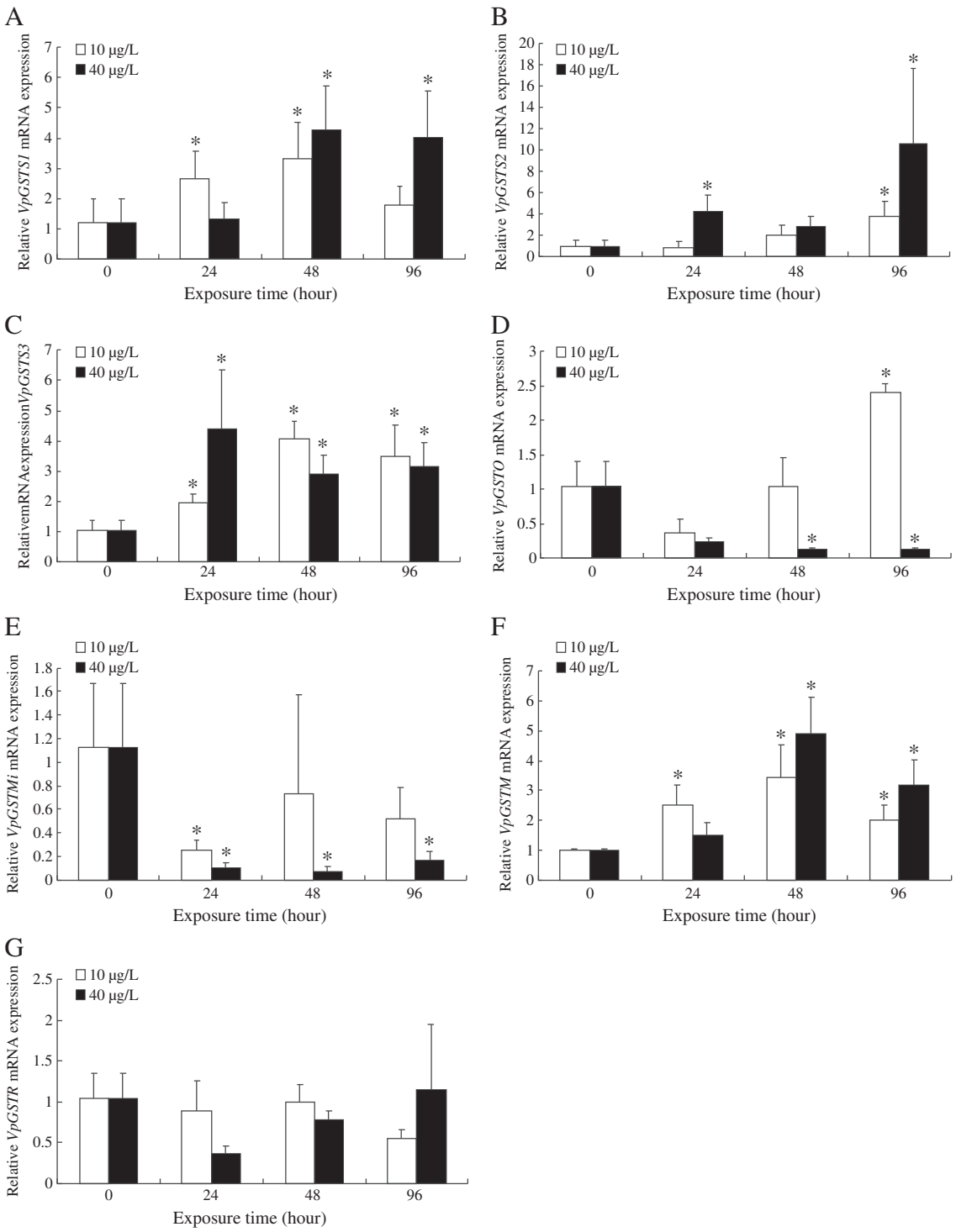


Fig. 5. Expression profiles of mRNA of (A) *VpGSTS1* (B) *VpGSTS2* (C) *VpGSTS3* (D) *VpGSTO* (E) *VpGSTMi* (F) *VpGSTM* and (G) *VpGSTR* after treatment with 10 and 40 μg/L Cu for 1 for 24, 48 and 96 h. *VpGSTs* mRNA expression level values were calculated relative to actin expression and shown as mean ± standard deviation ($n = 4$). Astericks indicate significant difference ($P < 0.05$) as compared to the control.

transcription of *VpGSTO* and *VpGSTMi* exposed to Cu. Additionally, these results indicated that *VpGSTs* showed higher susceptibility to Cu exposure than Cd, which was also reported in abalones (Wan et al., 2009) and intertidal copepod (Lee et al., 2008). It implied that the same dose of Cu caused more ROS production and subsequently severer oxidative damage. With regards to B[a]P exposure, the expression of *VpGSTS1* showed a significant decrease, while transcription of *VpGSTS2*, *VpGSTS3* and *VpGSTR* was highly induced. Our results suggested that *VpGSTs* displayed particularly high sensitivity to B[a]P exposure. Previous studies have also revealed that B[a]P exposure could induce GST activity and the expression of mRNA in *Mytilus galloprovincialis* (Hoarau et al., 2006; Banni et al., 2010) and *V. philippinarum* (Xu et al., 2010). Therefore, changes in *VpGSTs* expression observed in *V. philippinarum* exposed to B[a]P indicated that *VpGSTs* may have a defensive role against B[a]P toxicity. As an important phase II biotransformation enzyme, GSTs catalyze the

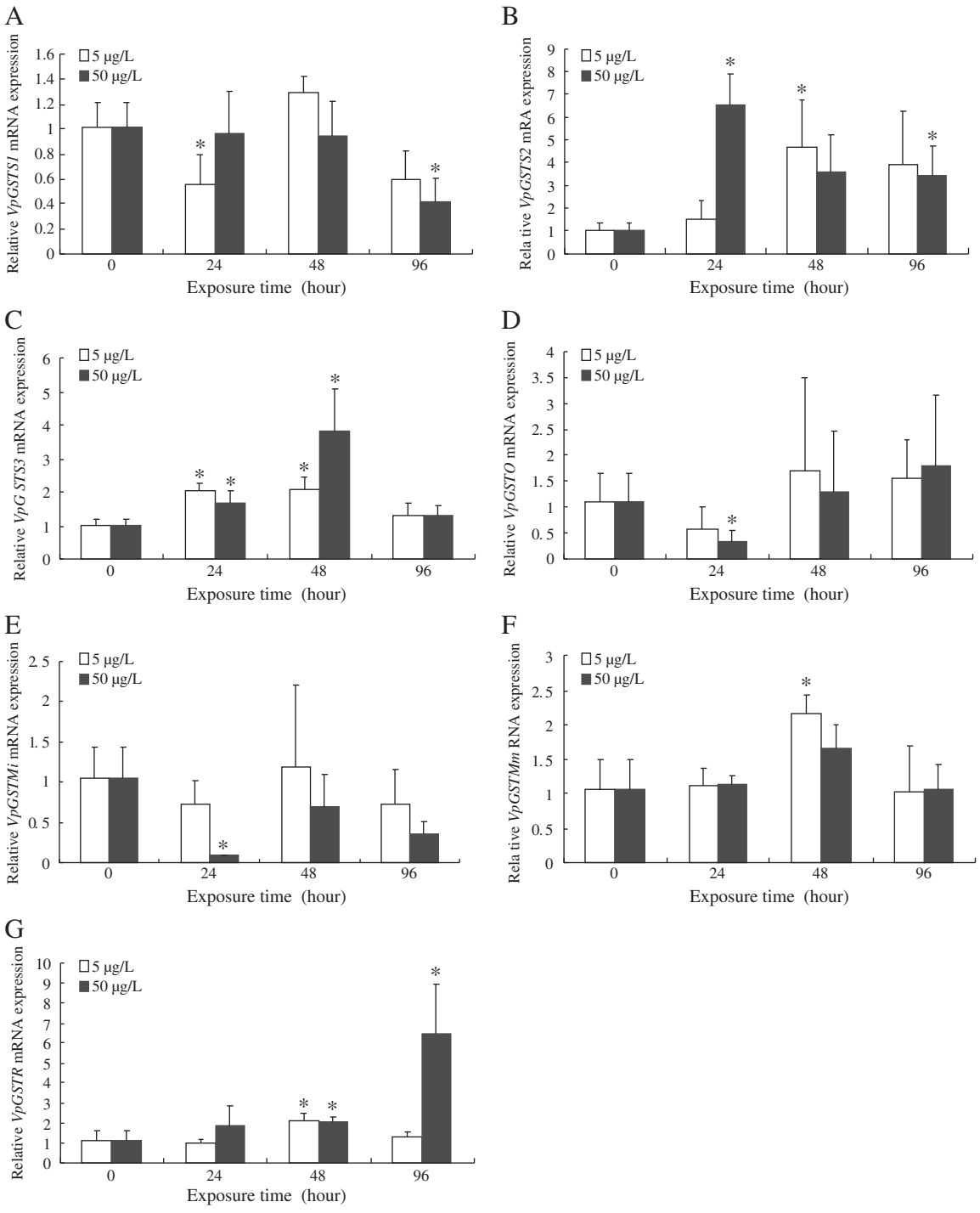


Fig. 6. Expression profiles of mRNA of (A) *VpGSTS1* (B) *VpGSTS2* (C) *VpGSTS3* (D) *VpGSTO* (E) *VpGSTMi* (F) *VpGSTM* and (G) *VpGSTR* after treatment with 5 and 50 μg/L B[a]P for 24, 48 and 96 h. *VpGSTs* mRNA expression level values were calculated relative to actin expression and shown as mean ± standard deviation ($n = 4$). Astericks indicate significant difference ($P<0.05$) as compared to the control.

conjugation of the glutathione (GSH) to electrophilic xenobiotics and oxidized components in order to increase their hydrophilicity and facilitate the excretion of toxicants (Ketterer et al., 1983).

5. Conclusions

In summary, we characterized seven *GST* genes in *V. philippinarum* and also examined their potential as biomarkers for heavy metals and B[a]P contamination. The expressions of *VpGSTs* transcript appeared to be selectively up- or down-regulated, which enhanced our understanding of the antioxidant roles of *VpGSTs* and their biomarker application. Of them, *VpGSTS2*, *VpGSTS3*, *VpGSTM* and *VpGSTR* exhibited significant up-regulation, indicating its good biomarker potential for Cu or B[a]P pollution in marine environment.

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