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Molecular cloning and differential expression patterns of sigma and omega glutathione S-transferases from *Venerupis philippinarum* to heavy metals and benzo[a]pyrene exposure*

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Abstract Glutathione S-transferases (GSTs) are a class of enzymes that facilitate the detoxification of xenobiotics, and also play important roles in antioxidant defense. We identified two glutathione S-transferase isoforms (VpGSTS, sigma GST; VpGSTO, omega GST) from *Venerupis philippinarum* by RACE approaches. The open reading frames of VpGSTS and VpGSTO were of 612 bp and 729 bp, encoding 203 and 242 amino acids with an estimated molecular mass of 22.88 and 27.94 kDa, respectively. The expression profiles of VpGSTS and VpGSTO responded to heavy metals and benzo[a]pyrene (B[a]P) exposure were investigated by quantitative real-time RT-PCR. The expression of VpGSTS and VpGSTO were both rapidly up-regulated, however, they showed differential expression patterns to different toxicants. Cd displayed stronger induction of VpGSTS expression with an approximately 12-fold increase than that of VpGSTO with a maximum 6.4-fold rise. Cu exposure resulted in similar expression patterns for both VpGSTS and VpGSTO. For B[a]P exposure, the maximum induction of VpGSTO was approximately two times higher than that of VpGSTS. Altogether, these findings implied the involvement of VpGSTS and VpGSTO in host antioxidant responses, and highlighted their potential as a biomarker to Cd and B[a]P exposure.

Keyword: *Venerupis philippinarum*; Glutathione S-transferase; mRNA expression; heavy metals; benzo[a]pyrene; biomarker

1I NTRODUCTION

Due to intense anthropogenic activities in recent decades, numerous contaminants including heavy metals (cadmium, copper, mercury, etc.) and polycyclic aromatic hydrocarbons (benzo[a]pyrene, dibenzanthracene, etc.) have been discharged into marine environment and posed severe threats to fragile marine ecosystem and even human beings through food chains (Rainbow, 1995; Xue and Warshawsky, 2006). To fully assess the impact of marine environmental pollutants, it is necessary to characterize the toxicological effects on resident biota caused by contaminations (Galloway et al., 2002). Exposure to environmental contaminants can lead to adaptive biochemical responses, and thus the response of some biotransformation enzymes may be considered biomarkers of aquatic pollution (Company et al., 2004; Nahrgang et al., 2009; Park et al., 2009). 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

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proteins, metallothioneins and vitellogenin have been frequently used for marine pollution monitoring (Cunha et al., 2005; Hoarau et al., 2006; Sarkar et al., 2006; Gao et al., 2007; Lee et al., 2008; Wang et al., 2009).

Glutathione S-transferases (GSTs, EC 2.5.1.18) constitute a large multigene family of phase II enzymes involved in detoxification of xenobiotics (DeJong et al., 1988). Based on the differences in structural, catalytic and immunological characters, at least 15 classes of GSTs (alpha, beta, delta, epsilon, kappa, lambda, mu, omega, phi, pi, sigma, tau, theta, zeta, and rho) have been identified from the cytosol, mitochondria and microsomes of numerous phylogenetically diverse organisms (Hayes et al., 2005). Generally, the detoxification mechanisms of GSTs lie in the conjugation of reduced glutathione with exogenous and endogenous toxic compounds or their metabolites to increase the hydrophilicity and facilitate the excretion of toxicants (Ketterer et al., 1983). The mRNA expressions of GST isoforms have been demonstrated to be inducible or repressed upon exposure to potentially toxic compounds, such as heavy metals, pesticides, tributyltin in diverse organisms (Boutet et al., 2004; Wang et al., 2006; Lee et al., 2008; Wan et al., 2008a, 2008b, 2009).

Manila clam *Venerupis philippinarum* is one of the important sentinel organisms in 'Mussel Watch Program' launched in China. Due to the wide distribution, high tolerance to salinity and temperature, ease of collection and high bioaccumulation of contaminants, *V. philippinarum* has been considered a good bioindicator in marine and coast ecotoxicology (Liang et al., 2004; Ji et al., 2006; Xu et al., 2010). Presently, several GST sequences have been identified from different mollusk species (Boutet et al., 2004; Wan et al., 2008a, 2008b, 2009; Ren et al., 2009; Xu et al., 2010). However, little information is available on the molecular features and expression profiles of GST in *V. philippinarum*. In this study, we cloned the full-length cDNA of sigma and omega GSTs from *V. philippinarum* (VpGSTS and VpGSTO) to compare their expression profiles exposed to three typical environmental contaminants in Bohai Sea and also to evaluate their potential as toxicological biomarkers.

2 MATERIAL AND METHOD

2.1 Clams and treatments

The clams *V. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local culturing farm (Bohai Sea, Yantai, China) and acclimatized for 10 days before exposures. The seawater was aerated continuously, and salinity and temperature maintained at 32 practical salinity units and 25°C throughout the experiment. Clams were fed with *Chlorella vulgaris* Beij daily and the seawater was renewed daily. After the acclimatization, the clams were randomly divided into twelve 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, 20, and 40 μg/L), Cu (as $CuCl_2$, 10, 20, and 40 μg/L) and B[a]P (5, 10, 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 Bohai Sea and the coastal waters of southeast China (Zhang, 2001; Zhang et al., 2004). The clams cultured in the normal filtered sea water (FSW) and FSW containing 0.002% DMSO (v/v) were used as the blank and control groups, respectively. The toxicant-laden seawater was renewed daily, and clams were fed daily during exposure time. Four individuals from each treatment were randomly sampled after exposure for 24, 48, and 96 h, respectively. The haemolymph from the control and the exposed groups was collected using a syringe and centrifuged at 2000×*g*, 4°C for 10 min to harvest the haemocytes.

2.2 Cloning the full-length cDNA of VpGSTS and VpGSTO

BLAST analysis of all expressed sequence tag (EST) sequences from the *V. philippinarum* haemocytes cDNA library revealed that two ESTs were highly similar to the previously identified sigma GST and omega GST. To generate the full-length cDNA of VpGSTS and VpGSTO, eight specific primers (Table 1) 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 (Zhang et al., 2011).

2.3 Sequence analysis

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

Table 1 Primers used in the present study

Primer	Sequence(5′-3′)	Sequence information
P1 (reverse)	TTTCTTTATGTCTGGTGCTG	5′ RACE primer for VpGSTS
P2 (reverse)	GTTCCCGCCGAGCTTTGATC	5′ RACE primer for VpGSTS
P3 (reverse)	CATAGGTTTGTCTCCTCCAA	5′ RACE primer for VpGSTO
P4 (reverse)	TGTCACAACCTTGGACCATA	5′ RACE primer for VpGSTO
P5 (forward)	GCTTGTTTTAGTGATTGCTGG	3′ RACE primer for VpGSTS
P6 (forward)	AACTTCGATCAAAGCTCGGCG	3′ RACE primer for VpGSTS
P7 (forward)	TCTCGGGTCAGGAAGTAAGT	3′ RACE primer for VpGSTO
P8 (forward)	TATCGGCATTAGATCGTGTC	3′ RACE primer for VpGSTO
P9 (forward)	CTGAGGAGGAACTTCTGATAG	Real time primer for VpGSTS
P10 (reverse)	GTTTCTTTATGTCTGGTGCTG	Real time primer for VpGSTS
P11 (forward)	ATGGTCCAAGGTTGTGACAC	Real time primer for VpGSTO
P12 (reverse)	CATCATAGGTTTGTCTCCTCC	Real time primer for VpGSTO

similarity and identity of VpGSTS and VpGSTO with GST proteins from other organisms were calculated by the Identity and Similarity Analysis program (http://www.biosoft.net/sms/index.html). Multiple alignments of VpGSTS and VpGSTO were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment show program (http://www.bio-soft.net/sms/index.html). A neighbor-joining (NJ) phylogenetic tree was constructed by MEGA 4.0 with 1 000 bootstrap replicates. Two phi GSTs from *Nicotiana tabacum* and *Arabidopsis thaliana* were used as the root of the tree.

2.4. Modulation of VpGSTS and VpGSTO gene expression exposed to Cd, Cu and B[a]P

The expression levels of VpGSTS and VpGSTO transcripts after Cd, Cu and B[a]P exposures were measured by quantitative real-time RT-PCR on an Applied Biosystem 7500 fast Real-time PCR System. Two sets of gene specific primers, P9 and P10 for VpGSTS and P11 and P12 for VpGSTO (Table 1), were designed to amplify products of 297 bp and 154 bp, respectively. These products was purified and sequenced to verify the PCR specificity. β-actin was used as a refrerence gene as previously described (Zhang et al., 2011), 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 RNA extraction, cDNA synthesis, reaction component, thermal profile, and the data analysis were conducted as previously described (Zhang et al., 2011). All datasets were given in terms of relative mRNA expression as means±S.D. 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. To compare mRNA expression between two exposure concentrations at the same time, the Student's t-test was used with significance at $P<0.05$.

3R ESULT

3.1 Sequence analysis of VpGSTS and VpGSTO

Two nucleotide sequences of 904 bp and 1 205 bp representing the complete cDNA sequence of VpGSTS and VpGSTO were obtained by RACE approach, respectively. These sequences were deposited in GenBank under accession No. HM061129 and HM061130. The deduced amino acid sequences of VpGSTS and VpGSTO were shown with the corresponding nucleotide acid sequence in Fig.1.

The open reading frames of VpGSTS and VpGSTO were of 612 and 729 bp, encoding 203 and 242 amino acids, respectively. The predicted molecular masses of VpGSTS and VpGSTO were 22.88 and 27.94 kDa. No signal peptide was identified in both VpGSTS and VpGSTO by signalP analysis, indicating that the deduced proteins should be cytosolic GST. CD-Search identified the GSH binding site (G-site, 4–73 aa for VpGSTS and 3–91 aa for VpGSTO) and non-specific xenobiotic binding site (H-site, 83–185 aa for VpGSTS and 109–226 aa for VpGSTO) in both VpGSTS.

Multiple alignment of sigma and omega GSTs revealed that their N-terminal domains were highly conserved, while the C-terminal domains were considerably variable (Figs.2, 3). Omega GSTs displayed higher sequence similarity across a broad spectrum of species compared with sigma GSTs. BLAST analysis showed that VpGSTS was homologous with Sigma-class GST of *Caenorhabditis elegans* (NP_496862). However, the degree of

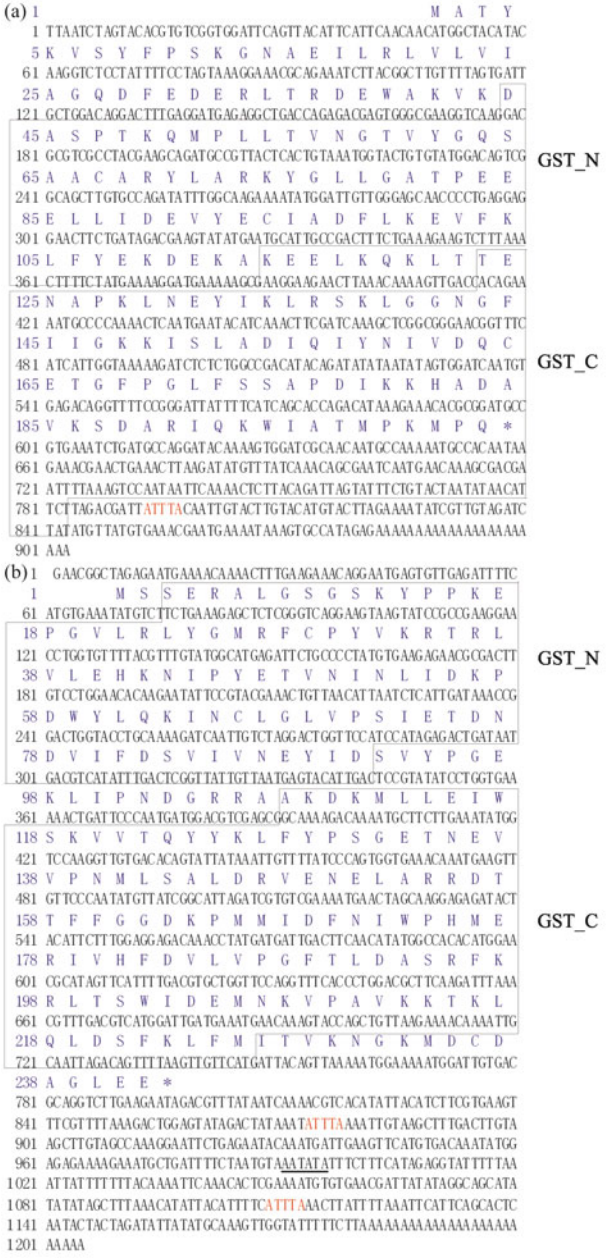

Fig.1 Nucleotide sequences and deduced amino acid sequences of VpGSTS (A) and VpGSTO (B)

The boxes show the conserved GST_N terminal and GST_C terminal domain. Polyadenylation signals are underlined and messenger stability determining motifs ATTTA are marked in red.

similarity was only 39%. It was observed that the VpGSTS was clustered together with another known *V. Philippinnarum* sigma GST sequence (ACU83216) on a branch of Sigma-class GST (Fig.4). From this tree, it was concluded that VpGSTS belonged to a distinctive category in the sigma family that has no close relationship with other origins. In contrast, VpGSTO fell into a group composed of abalone omega GSTs, oyster omega GST and *Pinctada fucata* GST (Fig.4). VpGSTO shared 44% identity with omega GST from *Haliotis discus discus* (ABO26600), 41% identity with *Crassostrea gigas* (CAD89618) and 40% identity with *P. fucata*(ADC35418).

3.2 Expression of VpGSTS and VpGSTO in response to heavy metals exposure

Quantitative real-time RT-PCR was performed to simultaneously assay the expression of VpGSTS and VpGSTO in response to heavy metals exposure. The results exhibited a differential expression pattern between VpGSTS and VpGSTO. For 10 μg/L Cd-treated group, the expression of VpGSTS mRNA increased significantly after exposure for both 48 and 96 h compared with the control group (Fig.5a). When the concentration increased to 20 and 40 μg/L, the significant inductions of VpGSTS were found throughout the experimental time. The maximal response appeared at 48 h (12.5-fold higher than the control) with the exposure of 40 μg/L Cd. After that, the expression of VpGSTS mRNA decreased to 3-fold of the original level at 96 h. For Cu exposure (Fig.5b), the expression levels of VpGSTS were significantly up-regulated in all exposed groups at 24 h (2.5-fold at 10 μg/L, 3.4-fold at 20 μg/L, and 2.3-fold and 40 μg/L). However, no significant difference was observed at 48 h of the Cu challenge groups. After 96 h exposure to Cu, level of VpGSTS mRNA expression was increased and significantly higher than the control group. On the whole, Cd displayed stronger induction of VpGSTS expression (approximately 12-fold) than that of Cu exposure (3.4-fold rise).

The transcriptional level of VpGSTO was also significantly induced by Cd exposure with fold change lower than VpGSTS (Fig.6a). For the concentration of 10 μg/L Cd, significant increase of VpGSTO expression was found at 96 h (6.4-fold higher than the control). The expression of VpGSTO significantly increased in 20 μg/L Cd-treated groups after exposure for 48 and 96 h. When the concentration increased to 40 μg/L, the inductions of VpGSTO were found throughout the experiment. Taken together, the results showed a certain time- and concentration-dependant increase in the expression of VpGSTO induced by Cd. As regards to Cu exposure, VpGSTO mRNA expression was significantly induced by all concentrations during the first 24 h, which was 3.6-, 4.3-, and 3.7-fold (10, 20, and 40 μg/L Cu) higher than the control, respectively (Fig.6b). After that, the expression level sharply dropped back to the original level.

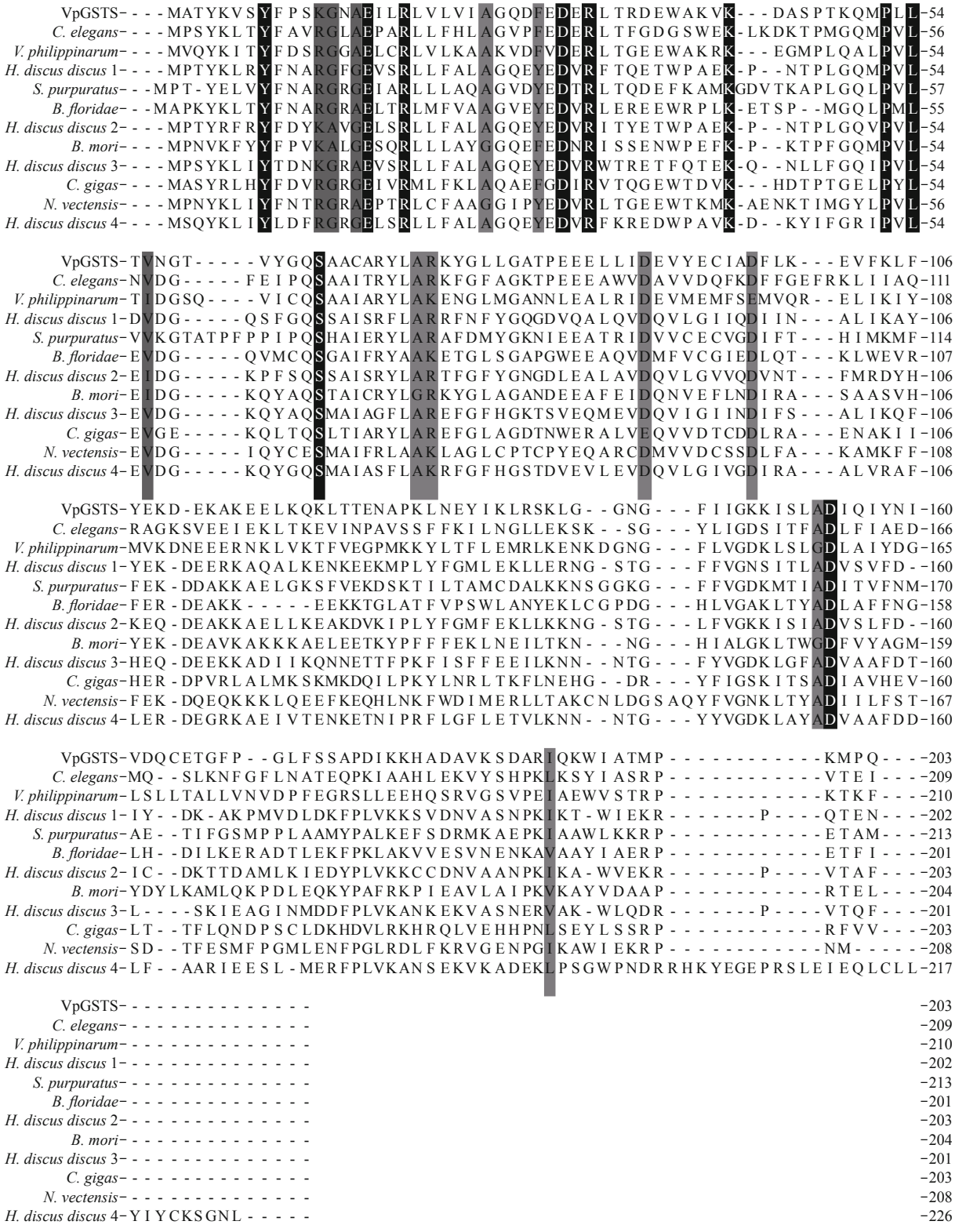

Fig.2 Alignment of amino acid sequences of VpGSTS and some similar protein sequences of GST class sigma or the like

The GST sequence from other organisms was obtained from GenBank database: *C. elegans* (*Caenorhabditis elegans*, NP_496862), *V. philippinarum* (*Ruditapes philippinarum*, ACU83216), *H. discus discus* 1 (*Haliotis discus discus*, ABO26603), *S. purpuratus* (*Strongylocentrotus purpuratus*, XP_791441), *B. floridae* (*Branchiostoma floridae*, XP_002610390), *H. discus discus* 2 (*Haliotis discus discus*, ABO26604), *B. mori* (*Bombyx mori*, NP_001036994), *H. discus discus* 3 (*Haliotis discus discus*, ABO26602), *C. gigas* (*Crassostrea gigas*, CAE11863), *N. vectensis* (*Nematostella vectensis*, XP_001637390), *H. discus discus* 4 (*Haliotis discus discus*, ABF67507). The amino acid residues with black background are totally conserved and the grey background are similarity amino acids.

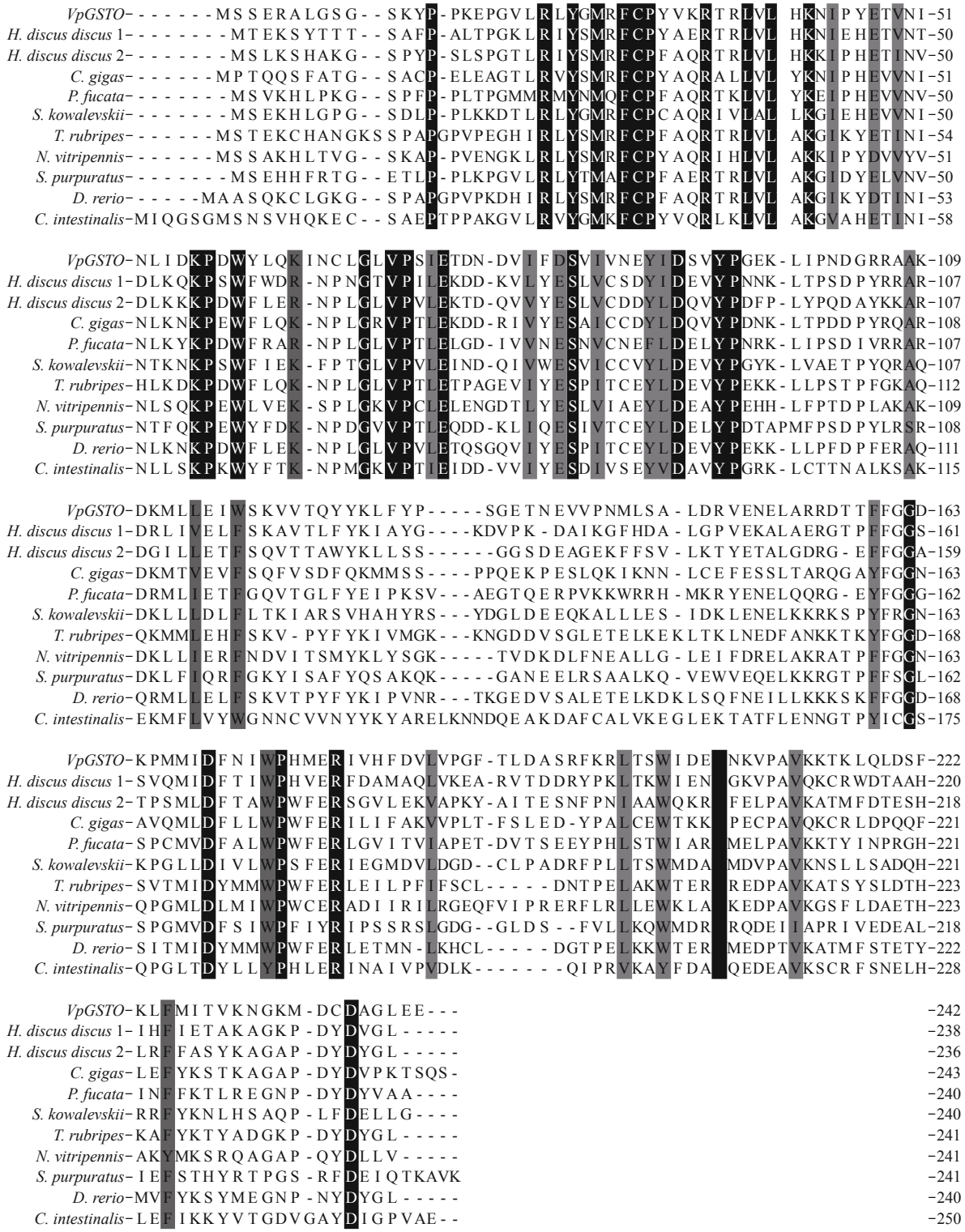

Fig.3 Alignment of amino acid sequences of VpGSTO and some similar protein sequences of GST class omega or the like

The GST sequence from other organisms was obtained from GenBank database: *H. discus discus* 1 (*Haliotis discus discus*, ABO26600); *H. discus discus* 2 (*Haliotis discus discus*, ABO26601), *C. gigas* (*Crassostrea gigas*, CAD89618), *P. fucata* (*Pinctada fucata*, ADC35418), *S. kowalevskii* (*Saccoglossus kowalevskii*, XP_002738514), *T. rubripes* (*Takifugu rubripes*, AAL08414), *N. vitripennis* (*Nasonia vitripennis*, NP_001165912), *S. purpuratus* (*Strongylocentrotus purpuratus*, XP_796486), *D. rerio* (*Danio rerio*, NP_001002621), *C. intestinalis* (*Ciona intestinalis*, XP_002122693), *H. discus discus* 4 (*Haliotis discus discus*, ABF67507). The amino acid residues with black background are totally conserved and the grey background are similarity amino acids.

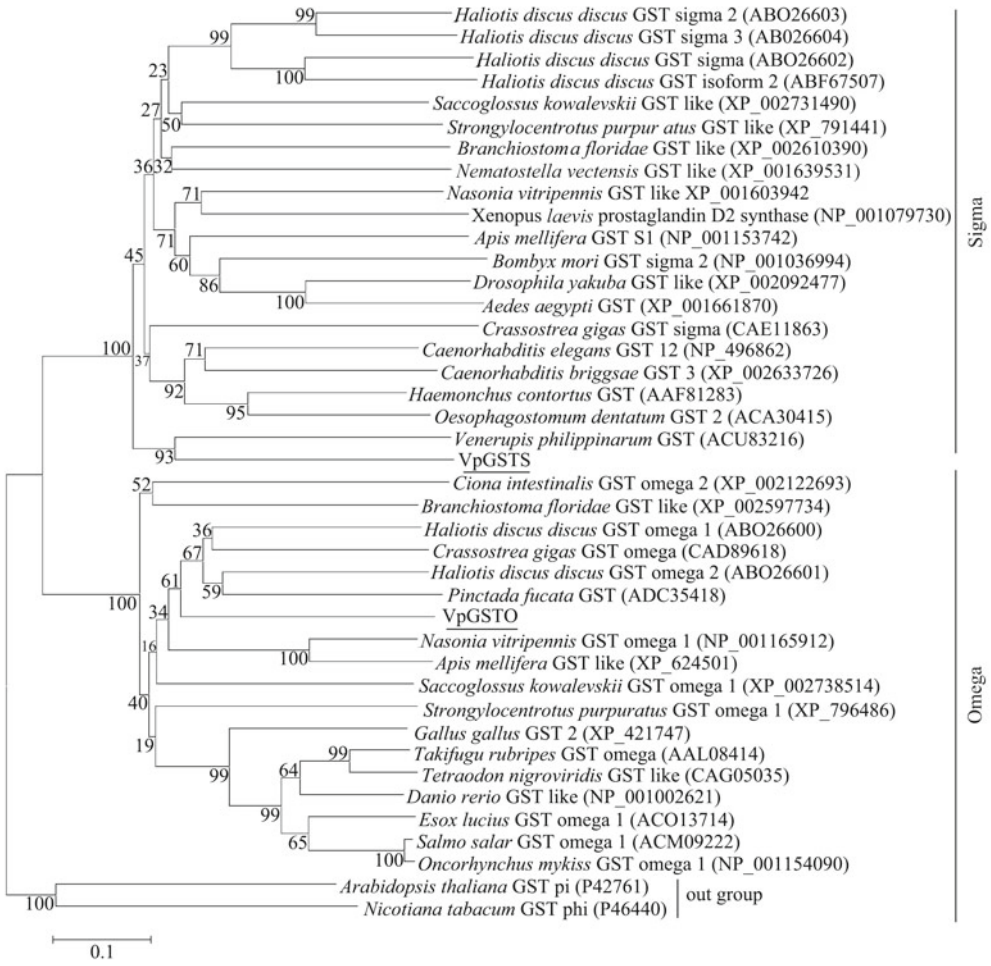

Fig.4 Phylogenetic tree constructed by the neighbor-joining method in MEGA software based on the GST sequences

VpGSTS and VpGSTO were underlined. Two GSTs of phi class (*Arabidopsis thaliana*, P42761 and *Nicotiana tabacum*, P46440) were used as out group. Numbers next to the branches indicated bootstrap value of each internal branch in the phylogenetic tree nodes from 1 000 replicates.

3.3 Expression of VpGSTS and VpGSTO in response to B[a]P exposure

After B[a]P exposure, both VpGSTS and VpGSTO were significantly induced at all concentrations during the first 48 h. Then some downward trend was observed at 96 h (Fig.7a). It was noteworthy that the fold change of VpGSTO expression post 10 μg/L B[a]P exposure was lower than that of 5 and 50 μg/L B[a]P-treated groups (Fig.7b) at 48 h. The maximal response of VpGSTS transcript was 6.0-fold greater than the control induced by 50 μg/L B[a]P, while the maximal response of VpGSTO in 50 μg/L B[a]P-exposed samples was as high as 14.2-fold to the control level. The maximum induction of VpGSTO by B[a]P was more than twice that of VpGSTS. Although the VpGSTS and VpGSTO expression in the blank and control groups were slightly fluctuant at different time points, no significant difference was found throughout the experiment (data not shown).

4D ISCUSSION

Recently, gene expression has been acknowledged as an effective approach to biomonitor environmental contamination in many marine mollusks (Boutet et al., 2003; Dondero et al., 2005; Wan et al., 2008b, 2009). Based on the mRNA expression profile data, it was possible to estimate chemical exposure and evaluate the risk of exposed contaminants (Lee et al., 2008).

Copper and Cadmium have been of great concerns in marine ecosystems since these two heavy metals could be bioaccumulated in the body tissues by marine organisms (Cunha et al., 2007). Exposure to Cu and Cd was shown to induce the expression of GST transcripts in many aquatic organisms (Yoshinaga et al., 2007; Lee et al., 2008; Kim et al., 2010). For example, expression of *Tigriopus japonicus* and *Takifugu obscurus* GSTs were significantly induced

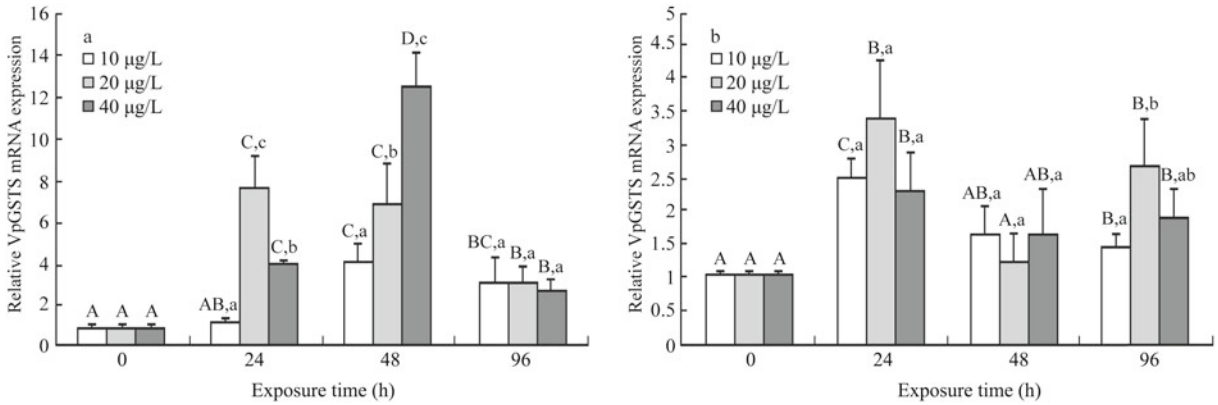

Fig.5 Temporal expression of VpGSTS transcripts in haemocytes of clams after exposed to different concentrations of Cd (a) and Cu (b)

Different small letters (a, b and c) indicated significant differences between different concentrations during the same sampling time. Different capital letters (A, B and C) indicated significant differences between time at the same concentration, Tukey's post hoc test $P<0.05$. Each bar represented mean±S.D. (n=4).

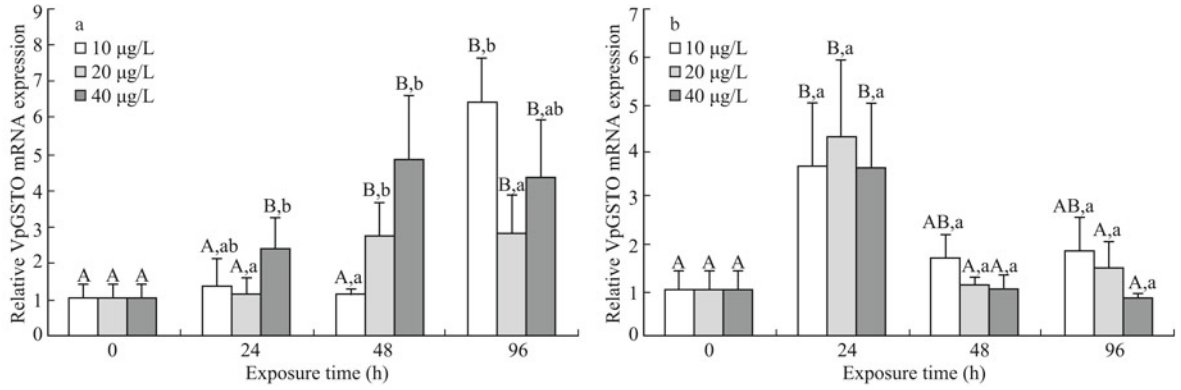

Fig.6 Temporal expression of VpGSTO transcripts in haemocytes of clams after exposed to different concentrations of Cd (a) and Cu (b)

Different small letters (a, b and c) indicated significant differences between different concentrations during the same sampling time. Different capital letters (A, B and C) indicated significant differences between time at the same concentration, Tukey's post hoc test $P<0.05$. Each bar represented mean±S.D. (n=4).

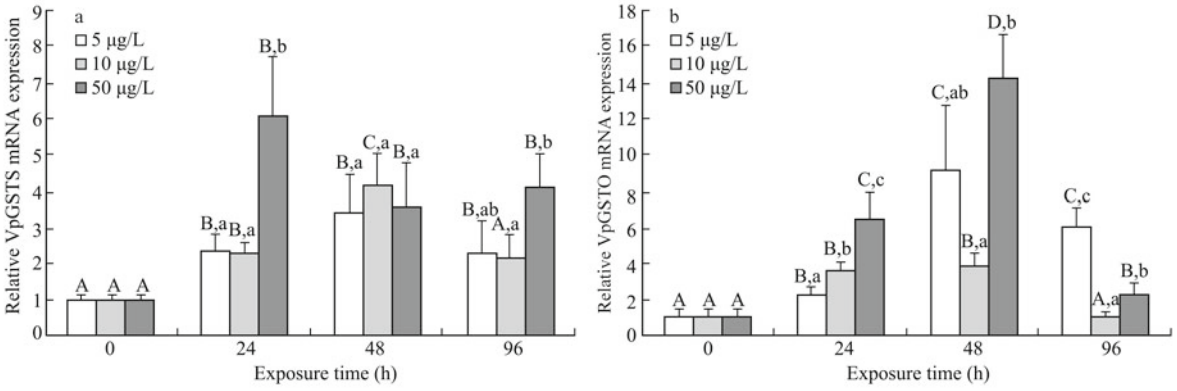

Fig.7 Temporal expression of VpGSTS (a) and VpGSTO (b) transcripts in haemocytes of clams after exposed to different concentrations of B[a]P

Different small letters (a, b and c) indicated significant differences between different concentrations during the same sampling time. Different capital letters (A, B and C) indicated significant differences between time at the same concentration, Tukey's post hoc test $P<0.05$. Each bar represented mean±S.D. (n=4).

by Cd and Cu exposure (Lee et al., 2007, 2008; Kim et al., 2010). In the current study, both VpGSTS and VpGSTO were significantly induced by Cd with larger amplitude in VpGSTS. Expression of VpGSTS transcript decreased obviously at 96 h, indicating the adaptation to the Cd exposure during the experimental period. However, VpGSTO expression level decreased slightly or even kept to increase at 96 h post Cd exposure. As concerned to Cu exposure, similar expression patterns were observed for VpGSTS and VpGSTO, with the highest induction in the early stage of challenge (24 h), and a gradual drop with ongoing Cu exposure. It was suggested that the decrease of VpGSTS expression level at 48 h might have resulted from the degranulation of VpGSTS producing haemocytes responding to Cu challenge. As time progressed on, the recruitment of VpGSTS producing haemocytes into circulating system probably contributed to the increase of VpGSTS transcript at 96 h. Although Cu is an essential element required by all organisms, it can be toxic in excess concentrations (Bryan and Hummerstone, 1971). A significant inhibition of GST activity was observed in *Tubifex tubifex* and *Cyprinus carpio* after exposure to 50–250 μg/L of Cu (Dautrememepuits et al., 2002; Mosleh et al., 2005). The expression of VpGSTS in Cd-exposed samples was much higher (12.5-fold) than that in Cu-exposed samples (3.5-fold), indicating that VpGSTS was more sensitive to Cd than to Cu. Meanwhile, the induction of VpGSTO in Cd-exposed group (6-fold) was somewhat higher than that of Cu-exposed samples (4.3-fold). Contrary to our results, Wan et al. (2009) reported that abalones GST-omega showed higher susceptibility to Cu than Cd. These findings highlighted the fact that specific GSTs response may vary with species and concentrations of toxicant (Blanchette et al., 2007).

B[a]P, as a potent carcinogen and/or mutagen, is usually oxidized by phase I mixed function oxidases to yield electrophilic intermediates, which are still toxic or become even more toxic in many cases (Wan et al., 2008b; Banni et al., 2010). As the most important phase II enzymes, GSTs have been extensively investigated as biomarkers for ecotoxicological assessment of organic pollutants (Boutet et al., 2004; Wan et al., 2008a, 2008b, 2009; Park et al., 2009; Banni et al., 2010). Previous studies have revealed that B[a]P exposure could induce B[a]P hydroxylase and GST activities in *Mytilus galloprovincialis* (Hoarau et al., 2006; Banni et al., 2010). Recently, a time-dependent increase in the expression of *V. philippinarum* GST-pi was induced by B[a]P and appeared a good linear correlation with B[a]P concentrations (0.01 and 0.2 μg/L) (Xu et al., 2010). In the present study, expression levels of both VpGSTS and VpGSTO were strongly induced by B[a]P exposure with more expression level for VpGSTO. These findings implied an detoxification role of VpGSTS and VpGSTO, and highlighted their potential as promising biomarker for B[a]P contamination in marine environment. Recently, several cis-acting regulatory sequences, such as antioxidant response elements (AREs) and xenobiotic response elements (XREs), have been found in the promoter regions of most GSTs (Wan et al., 2008b). It was assumed that contaminants exposure perhaps influenced transcription-regulating factors that bind to the response elements of some GST genes and enhance the transcription of relative GST isoforms in *V. phillipnurum*. Further investigations are needed to elucidate the response mechanism of *V. philippinarum* GST isoenzymes to contaminants exposure.

5C ONCLUSION

In the study, we cloned two class GST genes from *V. philippinarum* and investigated their temporal expression profiles after Cd, Cu, and B[a]P exposure. The expression of VpGSTS and VpGSTO were both rapidly up-regulated, however, they showed differential expression patterns to different toxicants. The differences in expression pattern of VpGSTS and VpGSTO indicated that these two isoenzymes probably play divergent physiological roles during the detoxification of various pollutants in *V. philippinarum*. All these findings highlighted their potential as a biomarker for indicating marine Cd and B[a]P contaminations.

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