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Gene cloning of a sigma class glutathione S-transferase from abalone (*Haliotis diversicolor*) and expression analysis upon bacterial challenge

Hong-Lin Ren [a,b,1], Dan-Dan Xu [a,1], Singaram Gopalakrishnan [a], Kun Qiao [a], Wei-Bin Huang [a], Ke-Jian Wang [a,*]

[a] State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen 361005, China
[b] Key Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, China

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ABSTRACT

Glutathione S-transferases (GSTs) are a multigene family of xenobiotic metabolizing phase II detoxification enzymes which take part in many pathological and physiological processes, and which can potentially be used as indicators and biomarkers for cancer diagnoses and organic or inorganic pollutant exposure. In this study, a full-length cDNA of a sigma class GST (*abGSTsigma*) (GenBank accession number EF546619) from variously colored abalone (*Haliotis diversicolor*) was identified. It was 1328 bp containing an open reading frame of 624 bp, encoding 208 amino acid residues with a predicted protein molecular weight of 23.67 kDa and an estimated p*I* of 5.67. Sequence analysis showed that the predicted protein sequence of *abGSTsigma* cDNA contained the conserved domain of the GST_N_Sigma_-like (PSSM: cd03039) and GST_C_Sigma_like (PSSM: cd03192). Alignment analysis demonstrated that the *abGSTsigma* of *H. diversicolor* was in a branch position with other known class sigma GSTs from different organisms. The *abGSTsigma* mRNA was distributed in multiple tissues tested and was highly demonstrated in the gill and mantle of normal abalones. In bacteria-challenged abalone, the *abGSTsigma* gene was significantly expressed in the hemocytes, gill, mantle and digestive gland and the total GSTs enzyme and SOD were also induced in the four tissues. The increased activities of SOD and GSTs can result in the elimination of reactive oxygen species (ROS) indicating antioxidant activities involved. The preliminary work revealed that the sigma class glutathione S-transferase gene *abGSTsigma*, a phase II detoxification enzyme, had a positive response to bacterial challenge, and that will lead to an insightful study on elucidating the interactions between immune responses and biotransformation exerted by *abGSTsigma*.

1. Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a well-characterized family of multifunctional isoenzymes that are ubiquitously distributed in bacteria, plants, and animals [1]. These isoenzymes represent about 1% of the total cellular proteins in eukaryotes and some prokaryotes, and belong to a group of phase II detoxification enzymes playing a crucial role in xenobiotic metabolism [2]. The primary function of GSTs is cellular defense against the toxicity induced by xenobiotic lipophilic compounds. GSTs also play a prominent role in many other physiological processes, including resisting oxidative stress [3], transporting endogenous hydrophobic compounds [2], catalyzing biosynthetic reactions [4] and acting as signaling molecules [5].

Previous studies found that GST expression levels are increased upon exposure to hydrogen peroxide and heavy metal [6], microcystin-LR [7], 4-nonylphenol [8], and endocrine-disrupting chemicals such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and tributyltin [9]. Although no criteria were clearly established to classify GSTs from marine organisms, the activities and transcript levels of GSTs have been widely investigated in some aquatic species as biomarkers upon exposure to organic or inorganic pollutants. Genes or proteins of GSTs are identified in some marine mollusks, such as the squid (*Ommastrephes sloani pacificus*) [10], blue mussel (*Mytilus edulis*) [11,12],

Abbreviations: 3-MC, 3-methylcholanthrene; *abGSTsigma*, *H. diversicolor* sigma class GST; CD, conserved domain; CDD, Conserved Domain Database; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferases; G-sites, GSH-binding sites; GlSTrs, partial cDNA of GST; HLS, hemocyte lysate suspension; H-sites, hydrophobic substrate binding sites; LPS, lipopolysaccharide; MW, molecular weight; NJ, neighbor-joining; ORF, open reading frame; qPCR, quantitative real-time PCR; PGDS, prostaglandin D_2 synthase; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; SOD, superoxide dismutase; SSH, suppression subtractive hybridization; sqRT-PCR, semi-quantitative reverse transcription PCR.

* Corresponding author. Tel.: +86 592 2184658; fax: +86 592 2180655.
E-mail address: wkjian@xmu.edu.cn (K.-J. Wang).

[1] These authors made equal contributions.

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quahog (*Mercinaria mercinaria*) [13], Mediterranean mussel (*Mytilus galloprovincialis*) [14], and disk abalone (*H. discus discus*) [9]. Previous study indicates that marine mollusks are widely used as indicator organisms in the marine environment [9].

In our previous study, one partial cDNA of GST (GlSTrs) was screened from the suppression subtractive hybridization (SSH) cDNA library of hemocytes in variously colored abalone challenged with bacteria [15]. It is known that GSTs play a major role in cellular defense against chemically induced toxicity, and few studies so far concerning the response of marine mollusk GSTs against bacterial infection [16]. Thus, this present study aimed to elucidate the full-length sequence of GlSTrs (*abGSTsigma*) and investigate the distribution of its transcripts in various tissues of normal abalones and the expression pattern in tissues of bacteria-challenged abalones. From this study we expect to provide new insight into the GST functions associated with immune processes other than xenobiotic metabolism.

2. Materials and methods

2.1. Experimental animals

Eighty live normal female *H. diversicolor*, averaging 55 ± 5 mm in shell length, obtained from the Zhangpu abalone farm in Fujian Province, were acclimated in the laboratory at a salinity of 3% for 7 days at 24 ± 1 °C seawater temperature, before experimentation. Animals were reared in 80 L PVC tanks containing 40 L natural seawater treated with sand filtration, kept on a natural daylight cycle and fed with the marine alga *Gracilaria tenuistipitata* during the acclimation and experimental period.

2.2. Bacterial challenge and preparation of different tissues

A mixed suspension of five bacterial strains including two Gram negative bacteria (*Escherichia coli* CGMCC 1.2389 and *Vibrio parahaemeolyticus* CGMCC 1.1615) and three Gram positive bacteria (*Staphylococcus aureus* CGMCC 1.89, *Micrococcus lysodeikticus* CGMCC 1.634 and *Staphylococcus epidermidis* CGMCC 1.2429), were prepared for the challenge experiments as described previously [15]. Abalones were each injected with a dose of 5×10^5 cfu in the front of the foot with 25 μL of stock bacterial suspension. Similarly, an equal volume of sterile saline solution (0.85% NaCl) was inoculated to each abalone as an unchallenged control. Three abalones at each challenge time point were arranged for the challenged and saline control groups, respectively. The abalones for each group were separately reared in individual tank under the same culture conditions. Meanwhile, three normal abalones were reared in an individual tank as a normal control group.

Sampling was performed at different time intervals (3, 6, 12, 24, 36, 48, 60, 72 and 84 h) after bacterial challenge. Hemolymph samples were collected by cutting the foot of each abalone from each group. Hemocytes were isolated by centrifugation at $800 \times g$, at 4 °C for 10 min and immediately kept in liquid nitrogen.

Samples from the gill, mantle, gonad, foot, epipodium, hypobranchial gland, radula, digestive gland, hemocytes, shell muscle, anus and kidney were separately collected from each individual abalone, and were individually frozen immediately in liquid nitrogen, and stored at −80 °C.

2.3. GST cDNA identification and generation of full-length cDNA

A cDNA clone, HDr3CJ41, with the homologous GST partial sequence was identified from the SSH cDNA library of hemocytes in *H. diversicolor* challenged with bacteria using BLASTp and BLASTx of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) [15].

The total RNAs were extracted from the hemocytes of the bacteria-challenged abalones using TRIZOL reagent following the manufacturer's instructions (Invitrogen) and were used for 5′- and 3′-rapid amplification of cDNA ends (RACE) cDNA synthesis with sense primers (3CJ41GST-S1 and 3CJ41GST-S2) and anti-sense primers (3CJ41GST-X1 and 3CJ41GST-X2) designed (Fig. 1) from the partial GST sequence (GlSTrs) identified in the SSH cDNA library. The RACE reactions were performed using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. 5′- and 3′-RACE products were purified from an agarose gel using a QiaQuick gel purification kit (Qiagen), ligated into the T/A cloning vector pMD18-T (TaKaRa) and transformed into *E. coli* X-Blue competent cells by heat shock. Three randomly selected clones were identified as positive clones using PCR and 1% agarose gel electrophoresis. The PCR reaction was performed using 1 μL bacterial culture, primers M13-47 and M13-48, and rTaq DNA polymerase (TaKaRa). The amplification conditions were: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C (annealing temperature), 90 s at 72 °C; then 3 min at 72 °C for further extension. Selected positive clones were sequenced at least twice using ABI 3730 automated sequencers (Applied Biosystems, USA) at Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (China).

2.4. Sequence analysis

The cDNA and predicted protein sequences were analyzed by means of GeneTool1.0 Lite, DNAStar5.0 (to deduce the amino acid sequence, to predict protein molecular weight (MW) and p*I*, and to calculate the percentage identity with the Clustal W program of MegAlign). Homology searches were performed using BLASTn and BLASTp in NCBI. The CD-Search service was used to identify the conserved domains (CDs) present in predicted protein sequences against NCBI's Conserved Domain Database (CDD, http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The deduced amino acid sequence of *abGSTsigma* was aligned with 30 known homologous proteins of GST class sigma obtained from GenBank using ClustalX1.83 software. The neighbor-joining (NJ) method was used to reconstruct a phylogenetic tree with 1000 bootstrap replicates by means of MAGA v4.0 software.

2.5. Analysis of gene expression pattern of abGSTsigma in H. diversicolor using semi-quantitative reverse transcription PCR and quantitative real-time PCR

In order to investigate the tissue distribution of GST transcripts, total RNA was extracted from various tissues of normal abalones, including the gill, mantle, gonad, foot, epipodium, hypobranchial gland, radula, digestive gland, hemocytes, shell muscle, anus and kidney tissues. In addition, to investigate the inducibility of GST transcripts in some tissues of *H. diversicolor* challenged with bacteria, total RNAs of the gill, mantle, digestive gland and hemocytes during the time course of bacteria-challenge were separately isolated from each tissue using TRIZOL reagent (Invitrogen). Complementary DNA was synthesized using the PrimeScript™ RT reagent kit (TaKaRa) following the manufacturer's instructions.

Total RNA of 50 ng for each sample of normal abalones was used as a template of semi-quantitative reverse transcription PCR (sqRT-PCR) to analyze the differential transcription of *abGSTsigma* from 12 tissues of normal abalones, using two specific primers 3CJ41GST-S1 and 3CJ41GST-X1. PCR reactions were performed in a final volume of 25 μL containing 0.375 μL dNTP mix (10 mM), 0.625 U Ex Taq (TaKaRa), 2.5 μL 10× Ex Taq buffer (Mg^{2+} plus), and 2.5 pmol of each gene-specific primer. The amplification conditions were: 1 min at 94 °C; 26 cycles of 30 s at 94 °C, 40 s at 60 °C

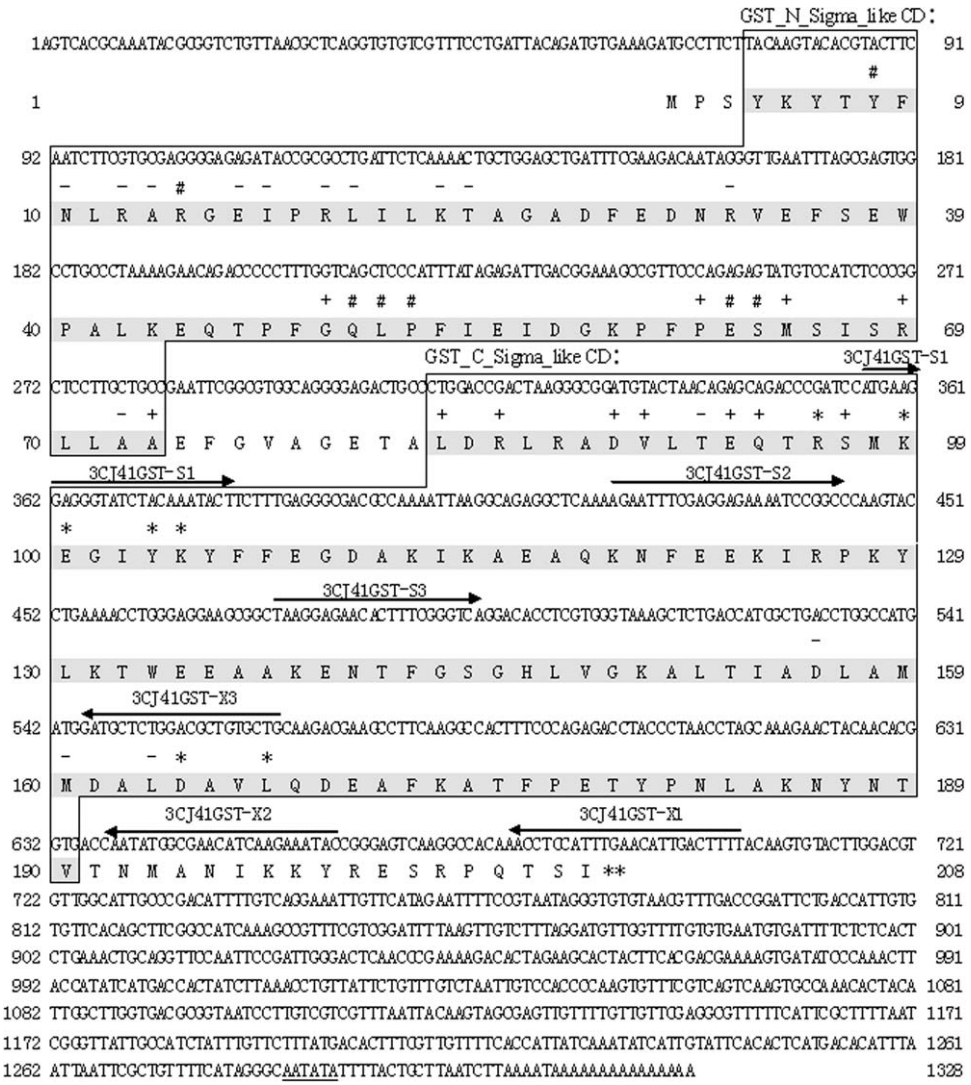

Fig. 1. Complementary DNA and predicted amino acid sequences of *abGSTsigma* from *H. diversicolor*. The polyadenylation signal is underlined and the stop codon is indicated by double asterisks (**). Primers for 5′-, 3′-RACE and analysis of gene differential expression binding sites are shown with arrows (5′ to 3′). The organization of the predicted conserved domains (CD) using the CD-Search service are framed including GST_N_sigma_like CD and GST_C_sigma_like CD. "#": GSH-binding sites (G-site) in N_terminal. "*": sites of substrate binding pocket (H-site) in C_terminal. "–": interacting interface sites of N_terminal domain with C_terminal domain. "+": dimer interface sites (GenBank accession number EF546619).

(annealing temperature), 40 s at 72 °C; then 7 min at 72 °C for further extension. The PCR product of 3 μL which was mixed with equal volumes from three abalones in the same condition was subjected to 1% agarose gel electrophoresis stained with ethidium bromide to evaluate expression levels in comparison with the internal standard housekeeping gene actin (GenBank accession number EF587284), which was obtained by sqRT-PCR with a pair of specific primers 4CJ11actin-S1: 5′-ACGGGTATTGTTCTT-GACTCTGGTG-3′ and 4CJ11actin-X1: 5′-TTTCTCCTTGATGTCCCT-GACGATT-3′ [15]. The intensities of amplified fragments were measured by Scion Image. Relative quantification of the GST expression level was calculated using the intensity value ratio of the target gene dividing actin gene.

Differentially expressed *abGSTsigma* transcripts after bacterial challenge were investigated by quantitative real-time PCR (qPCR), which was performed using the 7500 Real Time PCR System (Applied Biosystems). Total RNA was separately extracted from each tissue (gill, mantle, digestive gland and hemocytes) of the challenged, unchallenged control and normal abalones using TRIZOL reagent (Invitrogen). Two micrograms of total RNA from each group ($n = 3$) were separately reverse transcribed in a final volume of 40 μL using a PrimeScript™ RT reagent kit (Perfect Real Time) (TaKaRa) following the manufacturer's instructions. Real-time PCR was performed in a reaction mixture of 20 μL containing cDNA obtained from 10 ng of total RNA reverse transcribed, 5 pmol of each gene-specific primer (3CJ41GST-S3 and 3CJ41GST-X3) and 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, UK). The standard cycling conditions were 95 °C for 10 min (initial polymerase activation), followed by 40 cycles of 95 °C for 15 s (denaturation), 60 °C for 25 s (annealing), and 72 °C for 40 s (extending and fluorescence data collection). The PCR specificity was checked with a heat dissociation protocol from 60 to 95 °C. Data of raw relative quantification were calculated using the 7500 system SDS software version 1.3.1.21, using the housekeeping actin gene (specific primers 4CJ11actin-S2: 5′-ACCACGGGTATTGTTCTTGAC-3′ and 4CJ11actin-X2: 5′-CGGTGGTGGTGAAGGAGTAAC-3′) as the internal standard and normal group data as the calibrator. Statistical analysis of differences was done using SPSS 13.0 by one-way analysis of variance (ANOVA). Student's '*t*'-test was used to determine the differences between the challenged and saline control groups ($p < 0.05$).

2.6. Sample collection, tissue preparation and GST and SOD enzyme assays

Tissue samples were collected from each abalone of the challenged, saline control and normal groups ($n = 3$). Hemolymph was collected from individual abalone in a pre-chilled tube and the hemocytes were isolated by centrifugation at $800 \times g$, 4 °C for 10 min. The hemocytes suspended in PBS (100 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and a few crystals of phenylmethylsulfonyl fluoride, a protease inhibitor) were subjected to cell disruption by sonication (20 kHz, 50 W, 3× 20 s) in an ultrasonicator (Scientz JY92-II, Ning Bo Xinzi) and the resultant homogenates were centrifuged (12,000 × *g*, 30 min, 4 °C). Other tissue samples (gill, mantle and digestive gland) were individually homogenized (1:10, w/v) in PBS and then centrifuged for 30 min at 12,000 × *g* (4 °C). An aliquot of the resulting supernatant from each sample was used for determination of GST activity.

Glutathione S-transferase activity was assayed by the method of Habig et al. [17]. Briefly, the reaction was performed in a final volume of 1 mL containing 100 mM phosphate buffer of pH 6.5; 10 mM GSH reduced; 10 mM 1-chloro-2,4-dinitrobenzene (CDNB) and an appropriate amount of enzyme. Before addition of substrate, the enzyme mixture was incubated for 10 min at 37 °C and the reaction was initiated by addition of CDNB, and absorbance at 340 nm was monitored at 25 °C for 5 min. The changes in absorbance per minute were converted into moles of the substrate conjugated/min/mg protein using the molar extinction coefficient: $\varepsilon 340 = 9.6\ mM^{-1}\ cm^{-1}$ for CDNB.

Superoxide dismutase (SOD) activity was measured as the degree of inhibition of auto-oxidation of pyrogallol at an alkaline pH by the method of Marklund and Marklund [18]. One unit of SOD activity is defined as the amount of enzyme (per protein milligram) that inhibits the oxidation reaction by 50% of maximal inhibition.

Results are reported as mean ± S.D. of three observations per group and the data were subjected to one-way analysis of variance (one-way ANOVA) followed by Student's '*t*'-test. Differences between saline control and bacterial challenge groups were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Sequence analysis of the H. diversicolor abGSTsigma cDNA

The complete cDNA *abGSTsigma* (GenBank accession number EF546619) is 1328 bp, containing an open reading frame (ORF) of 624 bp with a coding capacity of 208 amino acid residues. The 3′ non-coding region is composed of 637 bp with a polyadenylation signal appearing at position 1286 nt and a poly(A) tail at position 618 nt downstream of stop codon TGA (Fig. 1). The predicted signal peptide cleavage site of its deduced protein was not found using the Signal P 3.0 Server (http://www.cbs.dtu.dk/services/SignalP), thus *abGSTsigma* might be a cytosolic glutathione S-transferase. The predicted MW of this protein was 23.67 kDa and its estimated p*I* was 5.67. The cytosolic GSTs in most organisms are all dimeric with subunit molecular masses from 21 to 29 kDa [19]. Our predicted MW of *abGSTsigma* was in accordance with a previous report that a sigma class GSTs show subunit MWs with an average of 23 kDa [1].

The complete cDNA sequence of *H. diversicolor abGSTsigma* was first reported in this study and showed less similarity to known sequences based on the BLASTn results. However, the predicted amino acid sequence of *abGSTsigma* was found to be homologous with the proteins of GST class sigma when analyzed by BLASTp with a creditable expectation value (*E*-value $\leq 10^{-3}$). On a search of the CD using the CD-Search service against NCBI's CDD, the predicted protein sequence of *abGSTsigma* cDNA was matched to CDs of the GST_N_Sigma_like (PSSM: cd03039) and GST_C_Sigma_like (PSSM: cd03192), containing a G-site (from Tyr^4 to Ala^{73}) which binds the GSH in the N-terminal region and an H-site (from Leu^{83} to Val^{190}) which is a substrate binding site in the C-terminal (Fig. 1). The sigma class GSTs rely on a Tyr (Y) residue for GSH stabilization in the G-site [1], which was also found in the sequence of *abGSTsigma* (Fig. 1). However, the similarity in the homologous proteins of the GST class sigma was relatively low, with a maximum identity of 38.7% in comparison with the GST-sigma of the African clawed frog (*Xenopus laevis*, AAM82563). The identity of *abGSTsigma* with each of eight class sigma GSTs from other organisms was at least 35%, which was shown as 36.6% identical with barber pole worm (*Haemonchus contortus*, AAF81283), 35.1% identical with red fire ant (*Solenopsis invicta*, ABA39530), 37.6% with *X. laevis* (NP_001079730), 35.9% and 35.7% with *Caenorhabditis elegans* (NP_494883 and NP_496357), 35.0% with African malaria mosquito (*Anopheles gambiae*, P46428), 37.6% with jewel wasp (*Nasonia vitripennis*, XP_001600977), and 36.9% with pig roundworm (*Ascaris suum*, P46436). The identities with class sigma GSTs of three mollusks, including disk abalone (*H. discus discus*, ABF67507), Pacific oyster (*Crassostrea gigas*, CAE11863) and squid (*O. sloani pacificus*, AAA92066) were 30.8%, 33.0% and 32.0%, respectively. The glutathione-dependent prostaglandin D_2 synthases (PGDSs) of chicken (*Gallus gallus*, O73888), rat (*Rattus norvegicus*, O35543), and human (*Homo sapiens*, O60760) are members of the sigma class GST [4,20,21], and their identities with *abGSTsigma* were 33.2%, 27.6%, and 28.1% respectively.

Based on the alignment analysis of the deduced protein sequences between *abGSTsigma* and 30 other GSTs with GST sigma CD including three PGDSs (GST class sigma), it was found that the N-terminal region of all the GSTs compared was highly homologous and conserved, while the C-terminal was relatively diverse (Fig. 2). Each GST is known to contain a G-site binding the GSH substrate in its N-terminal and an H-site binding the xenobiotic compounds in its C-terminal [22,23]. All of the members of the highly diverse GST super family are capable of binding the tripeptide GSH, thus it is suggested that the structural features of the G-site might share a highly conserved amino acid sequence. The H-site binding the xenobiotic compounds is the main structure accounting for speciality and activity of GSTs and it has few homologous amino acid sequences with that of the G-site. In general, the similarity of interclass protein sequences is rarely greater than 35% in the H-site region [1]. Xenobiotic compounds have various features, however the most important seems to be the possession of a carbon to carbon double bond bordering the electron-withdrawing group, which can be obtained through a phase I detoxification enzyme (cytochrome P450) if the compounds lack them intrinsically [24].

An NJ phylogenetic tree of *abGSTsigma* amino acid sequence with 30 GSTs containing GST sigma CD and 26 other class GSTs was reconstructed using MEGA v4.0 software. Three plant phi class GSTs of *Arabidopsis thaliana* (P42761) and *Nicotiana tabacum* (P46440 and BAA01394) were rooted to build the phylogenetic tree. It was shown that *abGSTsigma* of *H. diversicolor* was in a branch position with other known sigma class GSTs from different organisms, such as human, rat, African clawed frog, disk abalone, Pacific oyster, and *Tigriopus japonicus* (Fig. 3). GSTs are a multifunctional enzyme family, and the classification of the GST super family has been evolving to include cytosolic, microsomal and mitochondrial components [25]. Microsomal GSTs are less characterized and four classes of I–IV are identified [26]. The unique kappa class GSTs which belong to the mitochondrial GST isoenzymes have been investigated in mammalian species. This class has a very high peroxidase activity and their amino acid sequences are quite distinct from the other class cytosolic GSTs

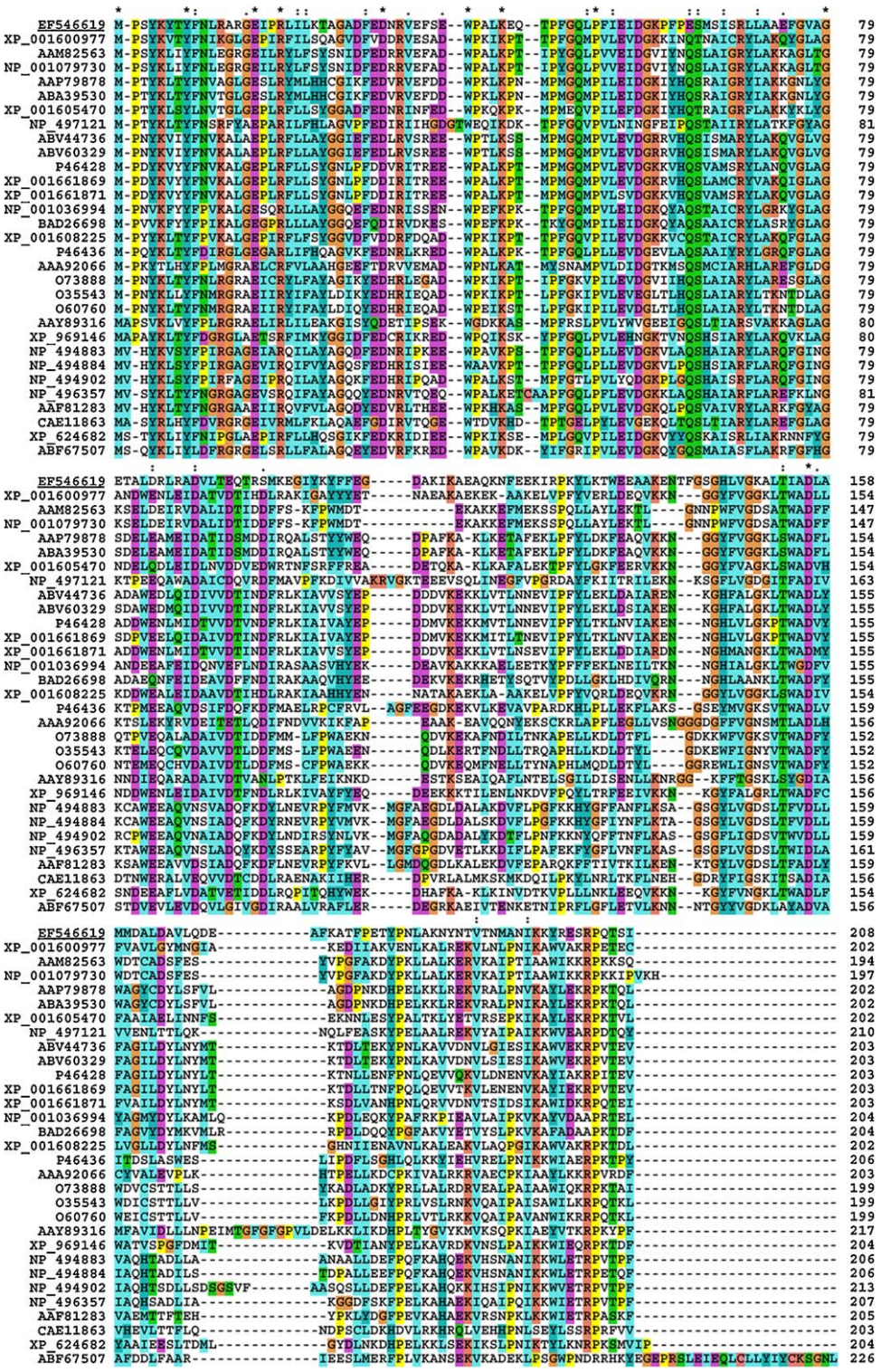

Fig. 2. Comparison of the amino acid sequence deduced from *H. diversicolor abGSTsigma* cDNA with some similar protein sequences of GST class sigma or the like. The aminoacid sequence deduced from *abGSTsigma* of variously colored abalone (*H. diversicolor*, GenBank accession number EF546619) is underlined. The conserved G-site of Tyr (Y) is indicated with the second asterisk present in the N_terminal of *abGSTsigma*. The aminoacid sequence of *abGSTsigma* in the N_terminal is relatively conserved. Other similar proteins are predicted and obtained from GenBank, as follows: jewel wasp GST class-sigma and GST isoform 2 (*Nasonia vitripennis*, XP_001600977, XP_001608225 and XP_001605470); African clawed frog GST sigma class (*Xenopus laevis*, AAM82563 and NP_001079730); red fire ant GSTS1 (*Solenopsis invicta*, AAP79878 and ABA39530); *Caenorhabditis elegans* GST-31, GST-7, GST-8, GST-30, and GST-5 (NP_497121, NP_494883, NP_494884, NP_494902, and NP_496357); *Phlebotomus papatasi* GST-like (ABV44736); *Lutzomyia longipalpis* GST1 (ABV60329); African malaria mosquito GST class-sigma (*Anopheles gambiae*, P46428); *Stegomyia aegypti* GST (*Aedes aegypti*, XP_001661869 and XP_001661871); domestic silkworm GST sigma (*Bombyx mori*, NP_001036994); diamondback moth GST 2-like (*Plutella xylostella*, BAD26698); pig roundworm GST class-sigma (*Ascaris sum*, P46436); Squid GST sigma (*Ommastrephes sloani pacificus*, AAA92066); chick PGDS or GST class sigma (*Gallus gallus*, O73888); rat PGDS or GST class sigma (*Rattus norvegicus*, O35543); human PGDS or GST class sigma (*Homo sapiens*, O60760); *Tigriopus japonicus* sigma class GST (AAY89316); red flour beetle GST-like (*Tribolium castaneum*, XP_969146); barber pole worm GST (*Haemonchus contortus*, AAF81283); Pacific oyster sigma class GST (*Crassostrea gigas*, CAE11863); honey bee GSTS1 isoform A-like (*Apis mellifera*, XP_624682); and disk abalone GST isoform 2 (*Haliotis discus discus*, ABF67507).

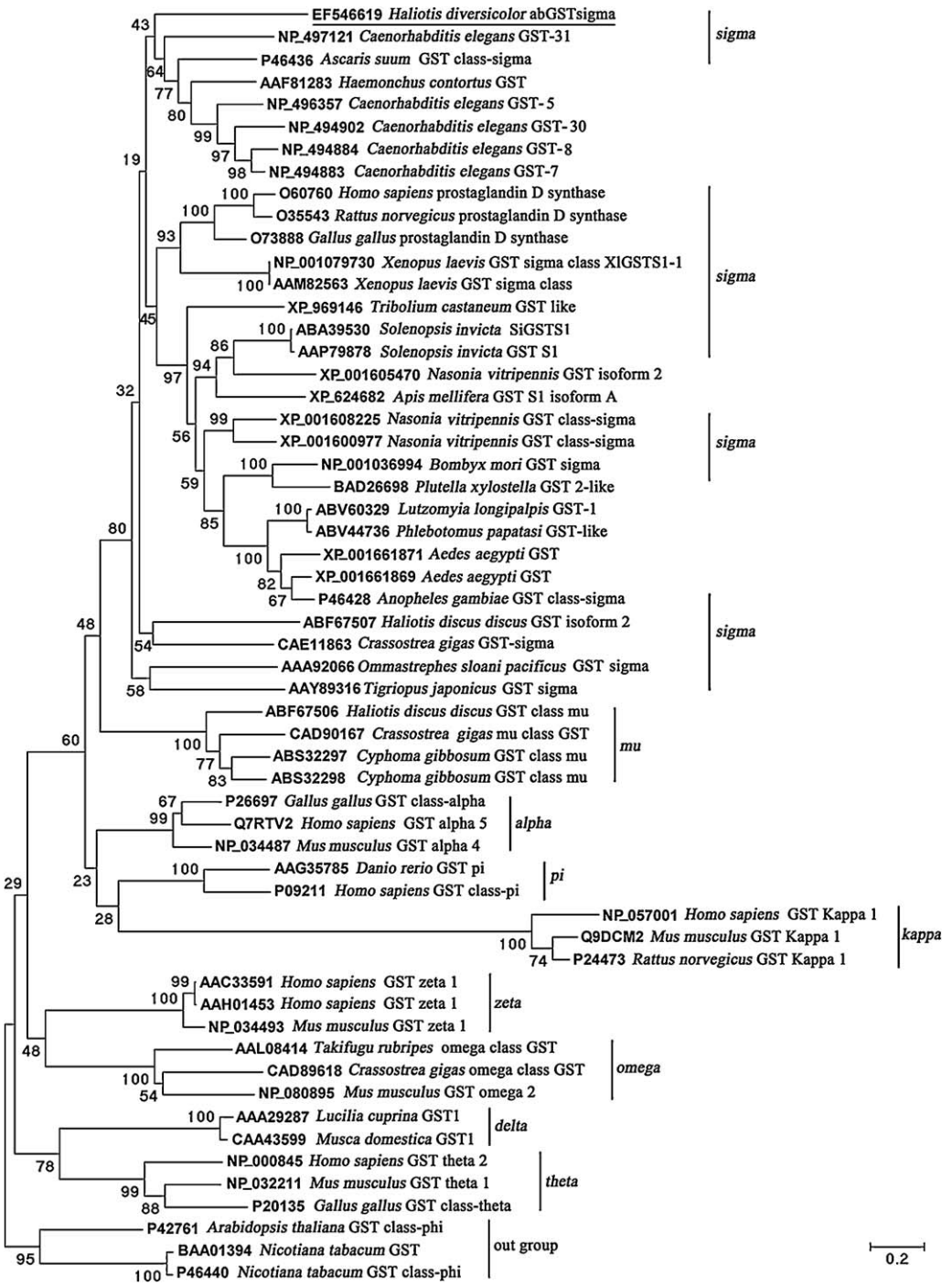

Fig. 3. Phylogenetic analysis of deduced amino acid sequences from *H. diversicolor abGSTsigma* and other GST cDNAs obtained from GenBank of the NCBI using MEGA v4.0. *H. diversicolor abGSTsigma* is underlined. Each class name of GST is marked. Neighbor-joining phylogenetic tree rooted with three plant GST including thale cress GST class (*Arabidopsis thaliana*, P42761), common tobacco GST and GST class-phi (*Nicotiana tabacum*, BAA01394 and P46440). Numbers next to the branches indicate bootstrap value of each internal branch in the phylogenetic tree nodes from 1000 replicates.

[25], which is shown in the NJ phylogenetic tree of *abGSTsigma* (Fig. 3). The classes of cytosolic GSTs are identified as alpha, mu, pi, sigma, theta, zeta and omega in mammals. Except for the sigma and omega classes, the rest are subdivided into subclasses [25]. So far, there are no clearly established criteria to classify GSTs from marine organisms [1]. In marine GSTs, the sigma class GSTs of the squid (*O. sloani pacificus*, AH003423) and *T. japonicus* (AAY89316) show little homology with other class GSTs [6,10]. Comparison of the sequence data indicates the unique nature of the GSTs from the sigma class, suggesting that it might play a role in physiological processes beyond its detoxification functions [1].

3.2. Differential tissue-specific expression of the abGSTsigma gene in normal abalones

Tissue-specific expression of *abGSTsigma* was analyzed using sqRT-PCR in multiple tissues of normal *H. diversicolor*, including both outer (gill, mantle, foot, epipodium, hypobranchial gland and anus) and inner (gonad, radula, digestive gland, hemocytes, shell muscle and kidney) tissues. Actin was used as an internal standard gene and the expression level of *abGSTsigma* in hemocytes was used as a calibrator contrasting with other tissues. The *abGSTsigma* cDNA fragment amplified from these tissues was 348 bp in length

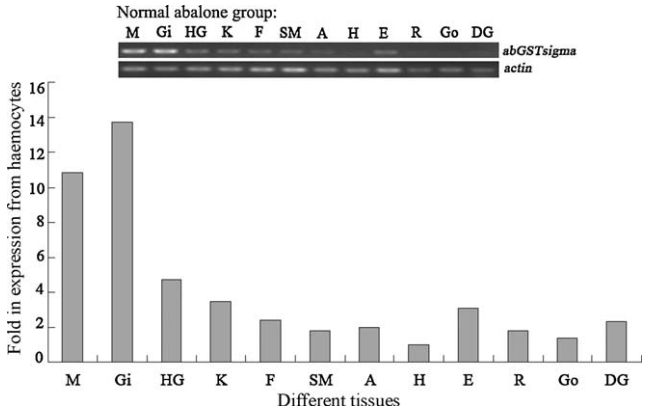

Fig. 4. Distribution of *abGSTsigma* mRNA transcripts in 12 tissues of normal variously colored abalone analyzed using RT-PCR. M, mantle; Gi, gill; HG, hypobranchial gland; K, kidney; F, foot; SM, shell muscle; A, anus; H, hemocytes; E, epipodium; R, radula; Go, gonad; DG, digestive gland.

(Fig. 4). As observed, the *abGSTsigma* gene expression was distributed in all tissues tested but the amount of transcripts in each tissue was very different. The high amount of *abGSTsigma* transcripts was demonstrated in the gill and mantle, and a relatively higher transcription level observed in hypobranchial gland, epipodium and kidney. In contrast, the *abGSTsigma* gene was low expressed in the other tissues tested, especially in hemocytes.

GSTs, as the most important components of xenobiotic metabolic enzymes of detoxification, are expressed with many patterns. Members of the GST super family exhibit different primary structures, enzyme properties, and physiological functions [27]. Each of the GST classes might have different distributions in tissues. To date, much research has investigated the tissue distribution of GSTs and analyzed the pathological and physiological functions of GSTs. Their expressions in marine organisms are generally abundant in the gill, digestive gland, gonad and mantle to protect organisms against the cytotoxic and or genotoxic effects of xenobiotics [8,9,28–30]. In shrimp GST class mu is expressed in the hepatopancreas and gills [28] and in disk abalone a high level of GST class mu (*HdGSTM1*) transcripts was observed in the gills and gonad, which may have a role in protecting tissues and gametocytes against endogenous and exogenous stress [9]. In addition, GST class sigma (XlGSTS1-1) of *X. laevis* is largely present in various amphibian tissues detected by Western blotting, including the liver, lung, heart, kidney and ovary. It is also indicated that XlGSTS1-1 might play an important role in protection against the toxicity of xenobiotics [30]. In our study, a high level of *abGSTsigma* transcripts was shown in the gill, mantle, hypobranchial gland, epipodium and kidney tissues, but was at a low level in the gonad and digestive gland of normal variously colored abalone. These results were somewhat different from the earlier studies, suggesting that *abGSTsigma* might be involved in some functions differing from the other class GSTs.

3.3. The expression pattern of abGSTsigma gene in tissues of H. diversicolor during the time course of bacterial challenge

Based on the results obtained by RT-PCR analysis, four tissues (the gill, mantle, digestive gland and hemocytes) of variously colored abalone were selected and the *abGSTsigma* gene expression pattern was evaluated in the four tissues after bacterial challenge using qPCR. Actin was used as an internal control gene. Saline solution was run in parallel at each time point of bacterial challenge in order to monitor the possible influence of saline solution on *abGSTsigma* expression. The mRNA level of *abGSTsigma* in each corresponding tissue of normal abalones was used as a calibrator to analyze the expression of *abGSTsigma* induced by bacteria.

As shown in Fig. 5, it was observed that the *abGSTsigma* gene was significantly expressed in tissues tested in comparison with the saline control after bacterial challenge. The *abGSTsigma* gene expression was induced not only in the outer tissues (gill and mantle) but also in the inner tissues (hemocytes and digestive gland). The gene expression pattern of *abGSTsigma* showed a similar trend among the four tissues. Two highest expression peaks of the *abGSTsigma* gene were observed during the period of bacterial challenge from 3 to 84 h, that is, the *abGSTsigma* gene expression was increased at 3 h after challenge in the hemocytes (Fig. 5A), gill (Fig. 5B), mantle (Fig. 5C) and at 12 h in the digestive gland (Fig. 5D), then the expression was induced to a markedly higher level in the four tissues at 24 h, and this we thought to be the first peak of gene expression. The highest amount of *abGSTsigma* transcripts in hemocytes, gill, mantle and digestive gland after 24 h of bacterial challenge showed approximately 45-fold, 25-fold, 15-fold and 25-fold increases compared to the normal group. It is very interesting to note that *abGSTsigma* gene expression returned to a lower level in the gill, mantle and hemocytes after 36 h postchallenge, and even more sharply down to a normal level in the digestive gland. After 48 h of bacterial challenge, the *abGSTsigma* mRNA level again increased and the second expression peak was observed around 60–72 h in the mantle, digestive gland and gill, while in the hemocytes the second highest expression level appeared around 48–60 h. Similarly as observed in gill and digestive gland at the time point of 36 h bacterial challenge, the *abGSTsigma* gene returned to a much lower level at 84 h after its highest peak. The expression level of the *abGSTsigma* gene in the hemocytes and mantle at the second peak was similar to those at the first peak, whereas in the digestive gland the expression level at the second peak around 60–72 h drastically increased and was nearly 80 times higher than at the first peak. In addition, it was also observed that the *abGSTsigma* gene could be induced to express when inoculated with saline solution, but the total expression level was much lower than that induced with bacterial challenge during the time course from 3 to 72 h with the exception of the gill at 24 h and the digestive gland at 3 and 60 h. Moreover, the gene expression induced with saline stimulation appeared higher than that induced by bacterial challenge at 84 h in all four tissues. Tissue-specific expression in normal abalones showed that the *abGSTsigma* transcripts were demonstrably higher in the gill and mantle than in the hemocytes and digestive gland (Fig. 4). Whereas, the *abGSTsigma* gene expression pattern, as described above postbacterial challenge, demonstrated that the inner tissues such as the hemocytes and digestive gland seemed to be more sensitive to the bacterial challenge, especially in the digestive gland as shown in Fig. 5 in which the *abGSTsigma* gene expression was highly increased to around 1960-fold the normal control at 60–72 h postchallenge. The reason may be related to the status of this animal. As is known, the aquatic environment is easily polluted with environmental xenobiotic compounds. GSTs are important phase II xenobiotic metabolism enzymes and induction of this type of enzyme would be a positive response of hosts protecting themselves from damage by chemically induced toxicity. In addition, there is a diversity of microorganisms existing in the marine environment and the outer tissues (gill and mantle) could also directly contact the environment in which some microorganisms already exist, thus potentially stimulating *abGSTsigma* gene expression in the gill and mantle, while the hemocytes and digestive gland are inner tissues which do not directly contact the environment and thus a lower response might be observed in normal animals. However under the conditions of bacterial challenge, the inner tissues are also

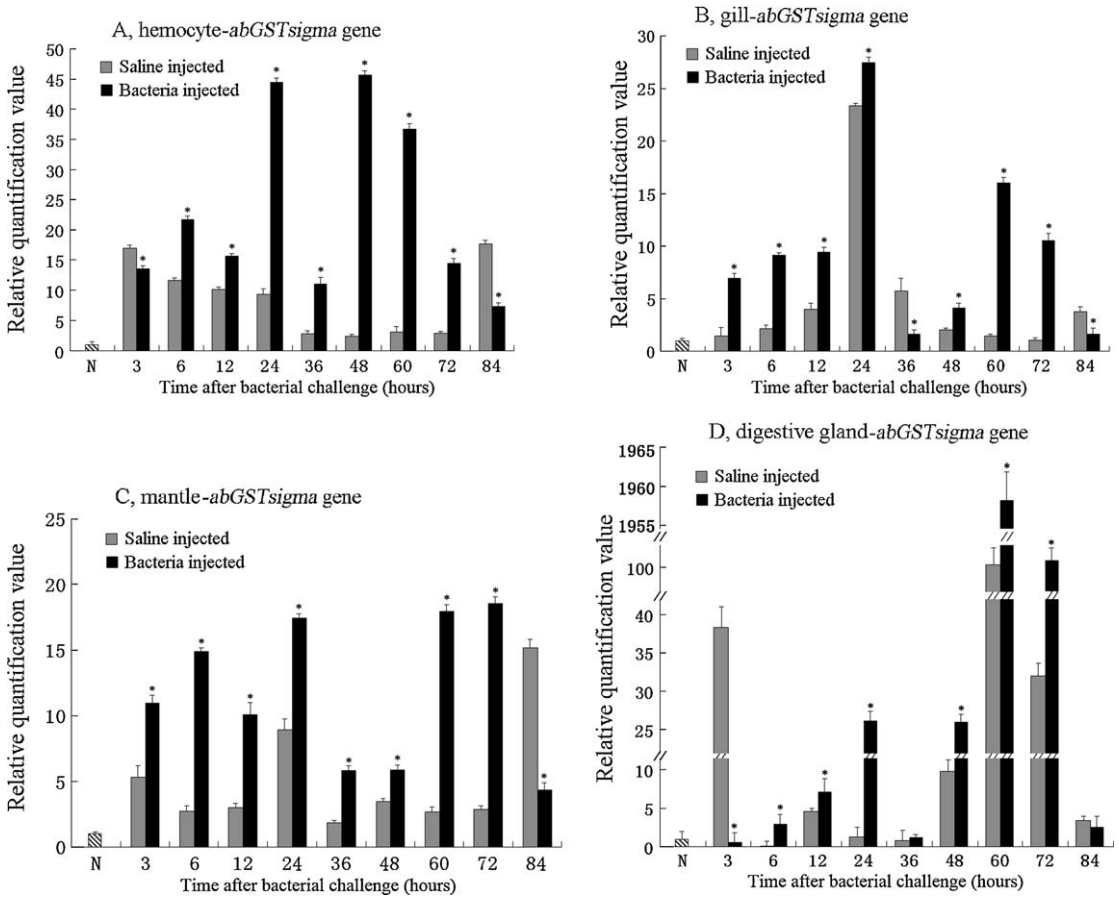

Fig. 5. Inducibility of *abGSTsigma* mRNA in hemocytes (A), gill (B), mantle (C) and digestive gland (D) of *H. diversicolor* during the time course of bacterial challenge analyzed using quantitative real-time PCR. "N": the normal groups. Statistical analysis of differences between the saline control and the bacterial challenged groups was done by one-way analysis of variance (ANOVA) using SPSS 13.0 software. An asterisk indicates that the difference is statistically significant at $p < 0.05$ levels.

stimulated by invading bacteria and thus the higher *abGSTsigma* gene expression detected in the present study.

GSTs are versatile functional enzymes. Sigma class GSTs play a crucial role in xenobiotic metabolism in the same way as other classes of GSTs. *T. japonicus* GST class sigma (GST-S) is up-regulated in response to exposure to two oxidative stress inducing agents, hydrogen peroxide (H_2O_2) and heavy metals (copper and manganese), suggesting that *Tigriopus* GST-S expression modulated by prooxidant chemicals may play a role against oxidative stress [6]. In a recent study three sigma GSTs (*HdGSTS1*, *HdGSTS2*, and *HdGSTS3*) of disk abalone are reported and it was found that *HdGSTS1* exhibits a proper inducibility by pollutants but the others reveal a minor role in the response of pollutants [31]. In our study, we found *abGSTsigma* expressions in hemocytes, gill, mantle, and digestive gland of *H. diversicolor* injected with bacteria, and the up-regulated *abGSTsigma* was induced with bacterial infection. The present data may support the hypotheses that the *abGSTsigma* had relationships with the immune event of bacterial infection and that the GST members of the multiple function enzyme super family might play a role in the immune functions of organisms in addition to xenobiotic metabolism. As a result, some putative factors such as reactive oxygen species brought forward from respiratory burst in the process of phagocytosis would be considered to be associated with the up-regulation of *abGSTsigma* in abalones challenged with bacteria besides other factors such as the metabolites produced by live bacteria, the endotoxin (such as lipopolysaccharide (LPS)) released from dead bacteria, and antibacterial immune-related substances synthesized. Previous studies show that there is an increase in oxygen consumption and then an increase in the production of reactive oxygen species (ROS) (which is the so-called respiratory burst) occurred during phagocytosis in animals [32]. The generation of ROS leading to oxidative damage will be interdicted by the antioxidant system in which several enzymes (superoxide dismutase, catalase and glutathione peroxidase) are subsequently induced as antioxidant defenses [33]. In the present study, the SOD activities were increased in the gill, mantle, hemocytes and digestive gland, at different challenge time points in the four tissues tested (Fig. 7). This result indicated that SOD could be induced upon bacterial challenge and its activity increase may account for the respiratory burst produced by bacterial invasion.

3.4. The induction of GST and SOD enzymes in tissues after bacterial challenge

The induction of the total GST enzyme known to be related to oxidative stress and antioxidant defense [6] was also investigated in the above mentioned tissues in order to evaluate whether the *abGSTsigma* gene and its related enzyme are correspondingly induced to express with the bacterial challenge. Total GST activity in each of the tissues was significantly higher when challenged with bacteria in comparison with that of the saline injected and the normal control. In the gill the highest activity was observed after 6 h of bacterial challenge and the increase in GST activity was statistically significant ($p < 0.05$) during the period of challenge except at 48 and 72 h (Fig. 6b). A similar increasing trend was observed for the mantle after bacterial challenge and the increase in GST activity was statistically significant ($p < 0.05$), but no

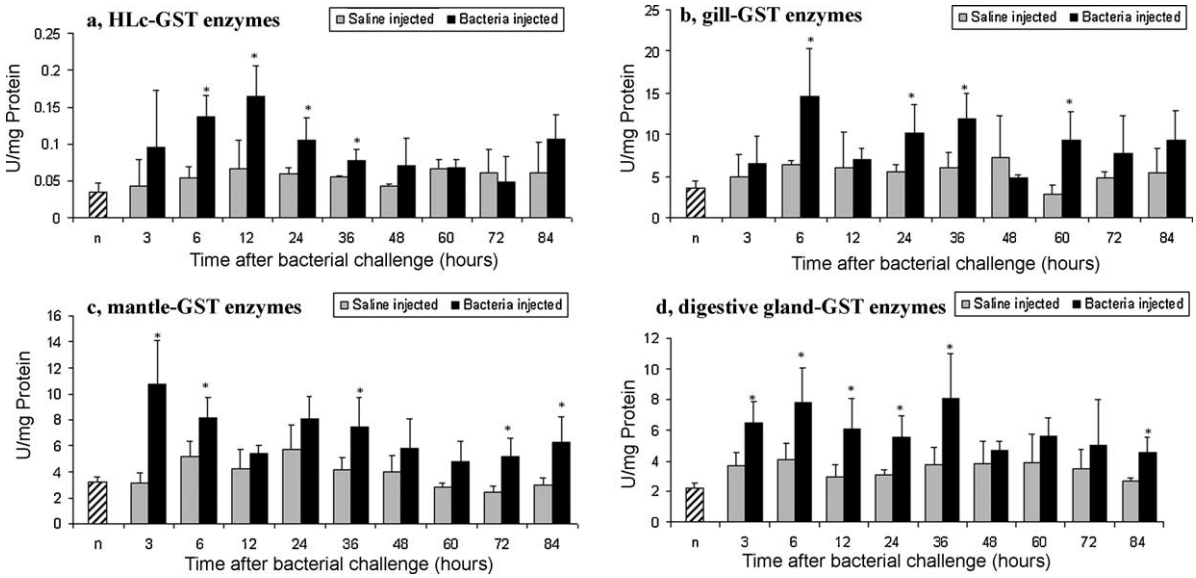

Fig. 6. Induction of the total GST enzyme in HLS (a), gill (b), mantle (c) and digestive gland (d) of abalone at different times during the course of bacterial challenge. "*n*": the normal groups. Student's '*t*'-test was used to determine the differences between the challenged and saline control groups. An asterisk indicates that the difference is statistically significant at $p < 0.05$ levels.

significant expression was detectable at 48, 60 and 72 h (Fig. 6c). In the digestive gland the highly GST activity was observed after 6 and 36 h of bacterial challenge and the increase in GST activity was statistically significant ($p < 0.05$) during all the time except 72 h of bacterial challenge (Fig. 6d). Although the HLS did not show significant increase for 3, 24, 48 and 72 h of bacterial challenge, the increase in GST activity after 6, 12, 36, 60 and 84 h of bacterial challenge also showed statistical significance ($p < 0.05$) when compared to the control (Fig. 6a). Moreover, the saline-injected groups showed significant increase only at 6 h in the gill and mantle and there was no significant increase detected in any of the tissues examined. Saline can induce homologous GST gene expression in that salinity changes induce alpha class GST mRNA up-regulation in the liver of the olive flounder (*Paralichthys olivaceus*) and increases GST activity in Mediterranean mussels (*M. galloprovincialis*) [34]. In the present study SOD activity in hemocytes and digestive gland of abalone was significantly induced before 36 h of postbacterial challenge (Fig. 7) and these results were more corresponding to those of total GST enzyme in the same tissue upon bacterial challenge (Fig. 6). It was also observed that the SOD activity in the gill and mantle significantly increased until 60 h of postbacterial challenge (Fig. 7), which was relatively corresponding to the total GST enzyme in the gill and mantle (Fig. 6). Induction of SOD and GSTs enzyme activities due to a bacteria *Galleria mellonella* infection was described in a recent publication [16]. Our results may be in agreement with Dubovskiy et al. [16] study and indicated that induction of SOD and GSTs enzyme may lead to the elimination of ROS generated due to bacterial challenge.

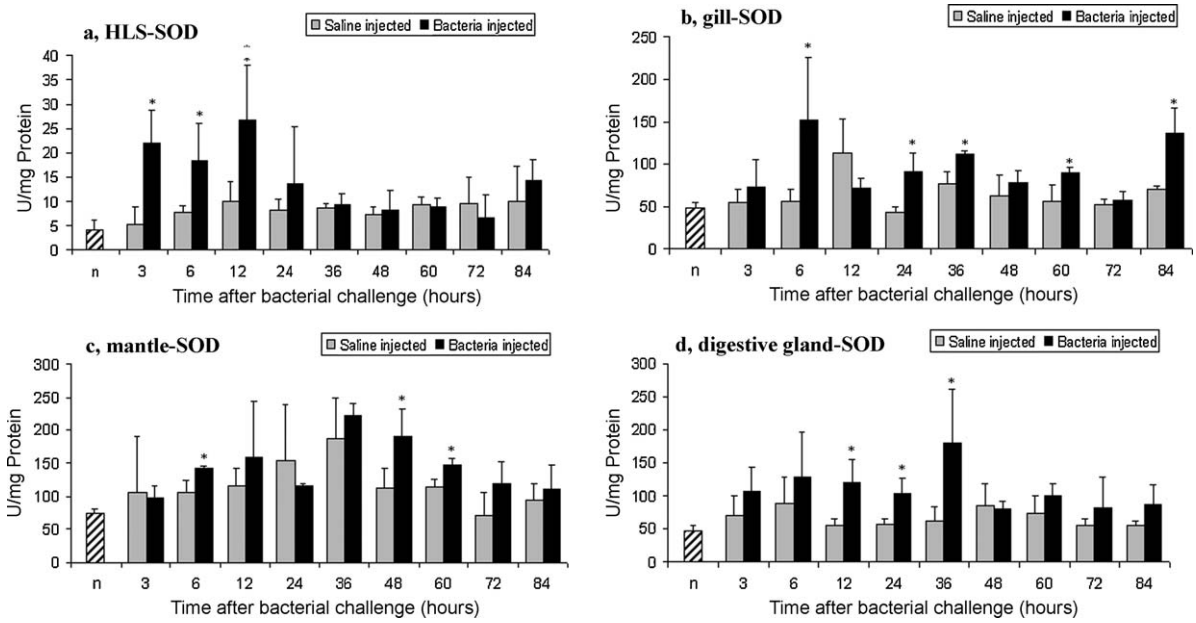

Fig. 7. Induction of SOD activities in HLS (a), gill (b), mantle (c) and digestive gland (d) of abalone at different times during the course of bacterial challenge. "*n*": the normal groups. Student's '*t*'-test was used to determine the differences between the challenged and saline control groups. An asterisk indicates that the difference is statistically significant at $p < 0.05$ levels.

Interestingly, we found that the activities of SOD and GSTs showed somewhat difference between the inner tissues (hemocytes and digestive gland) and the outer tissues (gill and mantle). The induction of two enzyme activities is relatively corresponding to *abGSTsigma* gene expression at the first peak, but in later stage (after 36 h of postbacterial challenge in hemocytes and digestive gland) these enzyme activities did not show significant induction whereas *abGSTsigma* gene expression was highly increased to a second peak after 48 h of postbacterial challenge. We noted that the activity of SOD and total GSTs enzyme was increased in the outer tissues (gill and mantle) even after 60 or 84 h of postbacterial challenge. Combined with the *abGSTsigma* gene expression we assumed whether the outer tissues are more prone to responding to the bacterial infection than the inner tissues, which is left to be studied further.

In our study, SOD and total GSTs enzyme were significantly induced in the gill, mantle, digestive gland and hemocytes of bacteria-challenged abalone. The significant induction of SOD and total GSTs activity further supported the presumption that the *abGSTsigma* gene expression in the study responding to the bacterial challenge might be associated with the antioxidant defense reaction. It is commonly known that a higher GST activity can imply a greater detoxification capacity [35,36], and so it can be postulated from our present study that *abGSTsigma* was not only involved in antioxidant defenses as a biotransformation enzyme to detoxify the damage in the digestive gland produced by bacteria, but was also probably involved in the direct response against bacteria invading the gill, mantle and hemocytes.

Interactions of immune systems and biotransformation have been demonstrated to be phylogenetically conserved from fish to mammals. In many cases, fish exposure to xenobiotics might be magnified with bacterial infection. The subsequent activation of the immune system can suppress detoxification activities and lead to increased mortality of fish [37]. In the present study, *abGSTsigma* was not down-regulated in *H. diversicolor* following injection with bacteria, differing from GST expression in the liver, spleen and head kidney of fish [38]. Knowledge concerning the immune defense and stress responses in abalone is scare and whether the sigma class GST of abalone plays an important role in the process of immune defense after bacterial challenge still waits to be further characterized.

4. Concluding remarks

A full-length cDNA sequence encoding an *abGSTsigma* has been characterized in this study, and *abGSTsigma* transcripts were widely distributed in various tissues tested of normal abalones. After bacterial challenge, the *abGSTsigma* gene and total GSTs enzyme were significantly expressed in the hemocytes, gill, mantle and digestive gland. The induction of SOD and GSTs resulted in the elimination of reactive oxygen species (ROS) indicating antioxidant activities involved in the innate immune defense. The present study indicated that the sigma class glutathione S-transferase *abGSTsigma*, a phase II detoxification enzyme, had a positive response to bacterial infection both in the outer and inner tissues of abalones. The findings from this study will provide new insights into the functions of GSTs and may lead to in depth studies concerning the potential interactions between immune reactions and biotransformation exerted by *abGSTsigma*.

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