Identification, genomic organization, and oxidative stress response of a sigma class glutathione S-transferase gene (*AccGSTS1*) in the honey bee, *Apis cerana cerana*

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Received: 3 October 2012 / Revised: 1 December 2012 / Accepted: 3 December 2012 / Published online: 20 December 2012
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Abstract Glutathione S-transferases (GSTs) are members of a multifunctional antioxidant enzyme superfamily that play pivotal roles in both detoxification and protection against oxidative damage caused by reactive oxygen species. In this study, a complementary DNA (cDNA) encoding a sigma class GST was identified in the Chinese honey bee, *Apis cerana cerana* (*AccGSTS1*). *AccGSTS1* was constitutively expressed in all tissues of adult worker bees, including the brain, fat body, epidermis, muscle, and midgut, with particularly robust transcription in the fat body. Relative messenger RNA expression levels of *AccGSTS1* at different developmental stages varied, with the highest levels of expression observed in adults. The potential function of *AccGSTS1* in cellular defenses against abiotic stresses (cold, heat, UV, H₂O₂, HgCl₂, and insecticides) was investigated. *AccGSTS1* was significantly upregulated in response to all of the treatment conditions examined, although the induction levels were varied. Recombinant *AccGSTS1* protein showed characteristic glutathione-conjugating catalytic activity toward 1-chloro-2,4-dinitrobenzene. Functional assays revealed that *AccGSTS1* could remove H₂O₂, thereby protecting DNA from oxidative damage. *Escherichia coli* overexpressing *AccGSTS1* showed long-term resistance

under conditions of oxidative stress. Together, these results suggest that *AccGSTS1* is a crucial antioxidant enzyme involved in cellular antioxidant defenses and honey bee survival.

Keywords Glutathione-S-transferase · Oxidative stress · Antioxidant defense · *Apis cerana cerana*

Introduction

Glutathione S-transferases (GSTs) belong to a large and diverse gene family of dimeric enzymes that are involved in the cellular detoxification of both natural and artificial molecules (Lumjuan et al. 2005; Ding et al. 2003; Rogers et al. 1999; Tang and Tu 1994). These enzymes are widely expressed in both prokaryotic and eukaryotic cells. Based on sequence identity, the mammalian GSTs are grouped into the following seven classes: alpha, mu, pi, theta, sigma, omega, and zeta (Flanagan and Smythe 2011). In insects, this highly diverse gene family falls into six major subclasses, including sigma, omega, theta, zeta, and insect-specific delta and epsilon (Hayes et al. 2005; Tu and Akgul 2005). GST enzymes exhibit remarkably broad and overlapping substrate specificities. They can catalyze the conjugation of the reduced glutathione (GSH) to electrophilic centers of a wide range of exogenous or endogenous toxic compounds, including cancer chemotherapeutic agents, chemical carcinogens, insecticides, herbicides, and oxidative stress products (Hayes et al. 2005). Some members also manifest glutathione peroxidase activity and promote the metabolism of organic hydroperoxides generated in cells (Ayyadevara et al. 2005; Burmeister et al. 2008; Singh et al. 2001; Tang and Tu 1994). As modulated expression levels of GST under oxidative stress are thought to represent

Electronic supplementary material The online version of this article (doi:10.1007/s12192-012-0394-7) contains supplementary material, which is available to authorized users.

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an adaptive response (Hayes and Pulford 1995), GST expression and activity are recognized as biomarkers of exposure to oxidative stressors (Durou et al. 2007; Nair et al. 2011).

The majority of reports on insect GSTs have concentrated on their roles in insecticide resistance via the detoxification of insecticides (Enayati et al. 2005; Lumjuan et al. 2011; Yang et al. 2009; Che-Mendoza et al. 2009). GSTs confer resistance by detoxifying lipid peroxidation products induced by pyrethroids, thereby protecting tissues from oxidative damage (Vontas et al. 2001), as well as by binding pyrethroid molecules in a sequestration mechanism, thereby offering passive protection (Kostaropoulos et al. 2001). Recently, studies on their roles in mediating oxidative stress responses have been extensively reported (Burmeister et al. 2008; Kampkotter et al. 2003; Li et al. 2008; Meng et al. 2009; Nair and Choi 2011; Umasuthan et al. 2012; Yang et al. 2012), and these studies have shown that GSTs reduce lipid hydroperoxides through a selenium-independent pathway. In *Chironomus riparius* fourth instar larvae, the upregulation of GSTs upon exposure to the pro-oxidative stress inducer paraquat indicates their involvement in oxidative stress defense mechanisms, while their induction upon exposure to cadmium and silver nanoparticles suggests a protective role (Nair and Choi 2011). In addition, Umasuthan et al. (2012) provided evidence that a *Ruditapes philippinarum* sigma-like GST could play a significant role in cellular defenses against oxidative stress caused by bacteria.

Because GST genes play important roles in detoxification pathways and the oxidative stress response, studies on GST resistance have been widely reported in insects, including *Drosophila melanogaster* (Li et al. 2008; Low et al. 2007; Sawicki et al. 2003; Tang and Tu 1994; Tu and Akgul 2005), *Anopheles gambiae* (Chen et al. 2003; Ding et al. 2003, 2005; Ranson et al. 2000, 2001), and *Bombyx mori* (Yamamoto et al. 2006; Yu et al. 2008), but information about other important species is rather limited. Honey bees, which serve as an excellent model for social behavior research, are a major plant pollinator and thus play essential roles in agriculture and ecological balance. Moreover, the Chinese honey bee, *Apis cerana cerana*, is suffering from decreasing populations size due to various environmental stressors including temperature, heavy metals, pesticides, and ultraviolet (UV) radiation (Xu et al. 2009), which are all known inducers of oxidative stress (Lushchak 2011; Kottuparambil et al. 2012). In this study, we report on the molecular cloning and characterization of a sigma class GST from *A. cerana cerana*. The expression profiles of *AccGSTS1* in response to various oxidative stresses were evaluated to establish the cellular defense function of this GST member against oxidative exposure. The potential roles of an *AccGSTS1* fusion protein in antioxidant defense were also investigated. Our results provide a better perspective on the mechanisms of resistance to oxidative stress in the honey bee.

Materials and methods

Experimental insects and treatments

Chinese honeybees (*A. cerana cerana*) used in the study were obtained from Shandong Agricultural University (Taian, China). Larvae and pupae worker bees were classified by the age, eye color, and shape. The larvae, pupae, and 1-day-old adult workers were taken from the hive, and the 2-week-old adult workers were collected at the entrance of the hive when they returned to the colony after foraging. Tissues from adults (15 days), including the brain, fat body, epidermis, muscle, and midgut, were dissected on ice. The midgut was separated under a stereomicroscope. Two-week-old adults were caged into groups of 30 and maintained in an incubator at a constant temperature (34 °C) and humidity (70 %) under a 24-h dark regimen. They were fed on a pollen-and-sucrose mixture before treatments. For cold and heat shock treatment, the temperature of the incubator was maintained at 4 and 43 °C, respectively. The UV light (30 mJ/cm²) was introduced to the incubator for UV treatment. For H₂O₂ treatment, honey bees were injected with 20 µl of H₂O₂ (50 µM of H₂O₂/worker) between the first and second abdominal segments using a sterile microscale needle. For metal treatment, groups of 30 adults were equally fed a mixture containing pollen and HgCl₂ (3 µg) with a micropipette. For generating pesticide resistance, three types of pesticides (phoxim, cyhalothrin, and acaricide) were diluted to a final concentration of 20 mg/l and fed to the worker bees. For the control, groups were fed only a pollen-sucrose solution. All honey bees were frozen in liquid nitrogen and stored at −70 °C until use.

RNA extraction, cDNA synthesis, and DNA preparation

Total RNA was extracted from the honeybees using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To eliminate genomic DNA contamination, each 2 µg sample of RNA was treated with 2 U of DNase I at 37 °C for 1 h. Then, complementary DNA (cDNA) was synthesized using the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. Genomic DNA was isolated from the whole bodies of adult bees using the EasyPure Genomic DNA Extraction Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions.

Isolation of the full-length cDNA sequence of *AccGSTS1*

Primers S1 and S2 were designed and synthesized (Shanghai Sangon Biotechnological Company, China) based on the conserved regions of the sigma class GSTs from other insects. These primers were then used to obtain

the internal region of *AccGSTS1*. Based on the sequence of the amplified fragment, two pairs of specific primers, 5R1/2 and 3R1/2, were subsequently designed and used for 5' and 3' rapid amplification of cDNA ends (RACE), respectively. The full-length cDNA sequence was determined by aligning the 5' and 3' UTR fragments with the internally conserved region. Another two primers, QC1 and QC2, were then synthesized to amplify the complete cDNA sequence of *AccGSTS1*. All primers and PCR amplification conditions used in this study are listed in Tables 1 and 2, respectively. All PCR products were separated by 1 % agarose gel electrophoresis, purified using a gel extraction kit (Solarbio, Beijing, China), ligated into pEASY-T3 vectors (TransGen Biotech, China), and transformed into *Escherichia coli* strain DH5 α competent cells. The positive clones were selected for sequencing.

Bioinformation analyses and phylogenetic characterization of *AccGSTS1*

Homologous *AccGSTS1* protein sequences were retrieved using the basic local alignment search tool (BLAST)

program from the NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and aligned using DNAMAN version 5.2.2 (Lynnon Biosoft, Quebec, Canada). Conserved domains (CDs) were identified using the PROSITE profile analysis (Bairoch et al. 1997) and CD database from NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). The molecular mass and isoelectric point of *AccGSTS1* was predicted using PeptideMass (http://web.expasy.org/compute_pi/). The tertiary structure was predicted using the online protein structure prediction tool SWISS-MODEL (<http://swissmodel.expasy.org/>). Phylogenetic analysis was performed using Molecular Evolutionary Genetic Analysis (MEGA) version 4.1 with the neighbor-joining (NJ) method.

Amplification of genomic sequence and copy number analysis

To obtain the genomic DNA sequence of *AccGSTS1*, two pairs of specific primers (SN1/SN2 and SN3/SN4) were designed and synthesized according to the full-length cDNA of *AccGSTS1*. PCR amplification was performed

Table 1 Primer sequences used in this study

Name	Primer sequence (5'-3')	Description
S1	TATTTTAATATTCCTGGTCTTG	cDNA sequence primer, forward
S2	GCATGTCAGTCAAAGATTCCTC	cDNA sequence primer, reverse
5R1	GCGATCAAGCGAGAAATAGCC	5' RACE forward primer, outer
5R2	GATTTAATCTTGGGCCATTCTTC	5' RACE forward primer, inner
AAP	GGCCACGCGTCGACTAGTAC(G) ₁₄	Abridged anchor primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged amplification primer
3R1	CCAAAGTTCCCTTGCTTCTC	3' RACE reverse primer, outer
3R2	GGAAAGTTGACGTGGGCAG	3' RACE reverse primer, inner
B26	GACTCTAGACGACATCGA(T) ₁₈	3' RACE universal primer, outer
B25	GACTCTAGACGACATCGA	3' RACE universal primer, inner
QC1	AGTCTGTGGAAGACCGTCTC	Full-length cDNA primer, forward
QC2	GTATCAAACGCGATTTAATG	Full-length cDNA primer, reverse
SN1	CAAAGAGGAGAGGTATGGTGG	Genomic sequence primer, forward
SN2	GTTGAGAAGCAAGGGAAC	Genomic sequence primer, reverse
SN3	GGCTATTTCTCGCTTGATCGC	Genomic sequence primer, forward
SN4	CCATAGATTGGGTGCGATTCTTC	Genomic sequence primer, reverse
GS-1	CCAAAGTTCCCTTGCTTCTCAAC	qPCR (copy number) primer, forward
GS-2	GCATGTCAGTCAAAGATTCCTC	qPCR (copy number) primer, reverse
GZ-1	CGAATAAAAGGGGAGGGAGGAG	Standard control primer, forward
GZ-2	CAGGCTTTGATGGACGGATTC	Standard control primer, reverse
SD-s	GGCTATTTCTCGCTTGATCGC	Real-time PCR primer, forward
SD-x	CGTAATCCACCACCTCTATCG	Real-time PCR primer, reverse
β -actin-s	GTTTTCCCATCTATCGTCGG	Standard control primer, forward
β -actin-x	TTTTCTCCATATCATCCCAG	Standard control primer, reverse
YH-F	GGATCCATGTCCACGTATAAATTGATT	Protein expression primer, forward
YH-R	GAGCTCAGATATGACCATAGATTGCG	Protein expression primer, reverse

Table 2 PCR amplification conditions in this study

S1/S2	10 min at 94 °C; 40 s at 94 °C, 40 s at 49 °C, and 50 s at 72 °C for 35 cycles; 5 min at 72 °C
5R1/AAP	10 min at 94 °C; 40 s at 94 °C, 40 s at 51 °C, and 1 min at 72 °C for 28 cycles; 5 min at 72 °C
5R2/AUAP	10 min at 94 °C; 40 s at 94 °C, 40 s at 48 °C, and 40 s at 72 °C for 35 cycles; 5 min at 72 °C
3R1/B26	10 min at 94 °C; 40 s at 94 °C, 40 s at 49 °C, and 1 min at 72 °C for 28 cycles; 5 min at 72 °C
3R2/B25	10 min at 94 °C; 40 s at 94 °C, 40 s at 52 °C, and 40 s at 72 °C for 35 cycles; 5 min at 72 °C
QC1/QC2	10 min at 94 °C; 40 s at 94 °C, 40 s at 45 °C, and 1 min at 72 °C for 35 cycles; 5 min at 72 °C
SN1/SN2	10 min at 94 °C; 40 s at 94 °C, 40 s at 43 °C, and 1 min 30 s at 72 °C for 35 cycles; 5 min at 72 °C
SN3/SN4	10 min at 94 °C; 40 s at 94 °C, 40 s at 51 °C, and 1 min 30 s at 72 °C for 35 cycles; 5 min at 72 °C
YH-F/YH-R	10 min at 94 °C; 40 s at 94 °C, 40 s at 50 °C, and 40 s at 72 °C for 35 cycles; 5 min at 72 °C

using the genomic DNA of *A. cerana cerana* as the template. The products were purified, cloned into pMD18-T (TaKaRa, Dalian, China), and transformed into competent *E. coli* DH5 α cells for sequencing. Two overlapping fragments obtained were spliced to form the *AccGSTS1* gene. The primers and reaction conditions are provided in Tables 1 and 2, respectively. The copy number of *AccGSTS1* in the honey bee genome was determined by real-time quantitative PCR, as described by Mason et al. (2002).

Real-time quantitative PCR

AccGSTS1 transcription was analyzed by quantitative PCR (qPCR) using the SYBR *Premix Ex Taq* (TaKaRa, Dalian, China) and the CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR mix was composed of 12.5 μ l SYBR *Premix Ex Taq*, 2.0 μ l of 1:10 diluted cDNA, 0.5 μ l of each primer (10 mM), and 9.5 μ l PCR grade water in a final volume of 25 μ l. The PCR reaction was carried out as follows: 1 cycle of 95 °C for 30 s; 40 cycles at 95 °C for 5 s, 56 °C for 15 s, and 72 °C for 15 s; and then a single melt cycle from 65 to 95 °C. The β -actin gene (GenBank ID XM640276), which is stably expressed (Yang et al. 2012; Umasuthan et al. 2012), was used as an internal control. Specific primers for *AccGSTS1* (SD-s, SD-x) and the reference gene (β -actin-s, β -actin-x) are listed in Table 1. Each sample was run in triplicate. The relative expression level of *AccGSTS1* was normalized to that of actin messenger RNA (mRNA) and was analyzed using CFX Manager software version 1.1 and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Overexpression and purification of recombinant AccGSTS1

The full-length cDNA of *AccGSTS1* was amplified using the primers YH-F and YH-R, in which a *Bam*HI site and a *Sac*I site were introduced, respectively. The PCR product was cloned into the prokaryotic expression vector pET-30a (+) and transformed into *E. coli* strain BL21 (DE3). The cells were grown in Luria–Bertani (LB) broth with kanamycin at 37 °C, and 1 mM final concentration isopropyl-1-thio- β -galactopyranoside (IPTG) was added to the cultures when the cell

density reached 0.4–0.6 OD₆₀₀. After expression, recombinant *AccGSTS1* was purified by Ni²⁺-nitrilotriacetate (Ni-NTA) resin (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The homogeneity of the enzyme preparation was analyzed by 15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were assayed by Coomassie Blue staining.

Determination of enzyme activity

Recombinantly expressed and Ni-NTA purified *AccGSTS1* was used for enzyme assays. GST activity was measured as previously described (Habig et al. 1974), with some modifications. Specifically, the reaction was performed in a 1-ml reaction system that contained 0.1 M sodium phosphate buffer (pH 6.5), 5 mM 1-chloro-2,4-dinitrobenzene (CDNB), 5 mM GSH, and an appropriate amount of purified recombinant protein. The change in absorbance at 340 nm for 1 min was converted into moles CDNB conjugated with GSH per minute per milligram of protein. The molar extinction coefficient (9.6 mM⁻¹cm⁻¹) for CDNB was used to convert absorbance into moles.

In vitro peroxidase activity

AccGSTS1 at different concentrations (0, 10, 20, 50, and 100 μ g/ml) was incubated in medium containing 100 mM Hepes buffer (pH 7.0) and 10 mM DTT at 37 °C for 10 min. The reaction (a total volume of 500 μ l) was initiated by the addition of 11.6 μ l of 30 % H₂O₂. After incubation for 0, 2, 5, and 10 min, 100 μ l of 100 % (w/v) trichloroacetic acid was added to stop the reaction. Then, 200 μ l of 10 mM Fe(NH₄)₂(SO₄)₂ and 100 μ l of 2.5 M KSCN were added, resulting in the red-colored ferrithiocyanate complex. The remaining peroxide content was determined by measuring the absorbance decrease at 475 nm.

DNA cleavage assay

The DNA cleavage assay was performed using the mixed-function oxidation (MFO) system that can cause DNA damage. The reaction mixture (25 μ l) containing 2 μ g of

supercoiled pUC19 plasmid DNA (TaKaRa, Dalian, China), 3 μ M FeCl₃ and 10 mM DTT in 100 mM Hepes buffer (pH 7.0) and increasing concentrations of AccGSTS1 was incubated at 37 °C for 3 h and was then subjected to 1 % agarose gel electrophoresis for the determination of DNA cleavage.

Disc diffusion assay

E. coli BL21 (DE3) cells were cultured in LB broth with kanamycin at 37 °C until the cell density reached 0.4–0.6 OD₆₀₀, then IPTG was introduced to the medium at a final concentration of 1 mM. After being induced for 6 h, approximately 5×10^8 cells overexpressing recombinant AccGSTS1 were overlaid onto LB–kanamycin agar and incubated at 37 °C for 1 h. Cells with the pET-30a (+) vector only were used as the control. Filter discs (6 mm in diameter) soaked in different concentrations of paraquat, cumene hydroperoxide, and *t*-butylhydroperoxide were placed on the surface of the top agar and incubated at 37 °C for 24 h, and then the killing zones around the discs were measured (Burmeister et al. 2008).

Statistical analysis

The gene expression pattern and in vitro experimental data were subjected to Duncan's multiple range test with an analysis of variance (ANOVA) using Statistical Analysis System (SAS) version 9.1 software. Comparisons showing significant differences are shown.

Results and discussion

Isolation and molecular characterization of AccGSTS1

The full-length cDNA sequence of a Sigma class GST (*AccGSTS1*) from *A. cerana cerana* was obtained using a combination of reverse transcription PCR (RT-PCR) and RACE and was deposited in GenBank with the accession number HQ828076. This sequence encompasses a 615 bp open reading frame (ORF) that encodes a 204-amino acid residue protein, in addition to a 5'-untranslated region (UTR) of 60 bp and a 3'-UTR of 163 bp. No *trans*-splicing was observed at the 5' end of the transcripts obtained by RACE. The AccGSTS1 protein has a predicted molecular mass of 23.737 kDa and a theoretical isoelectric point of 8.75. The predicted molecular mass is consistent with other sigma class GSTs, which have an average molecular mass of 23 kDa (Blanchette et al. 2007).

Distribution of sigma class GSTs in the honey bee differs substantially from that in the fruit fly and mosquito; there are four sigma class GSTs in *Apis mellifera*, indicating gene expansion (Corona and Robinson 2006). Our previous study

isolated one sigma class GST gene from the Chinese honey bee, *AccGSTS4* (Yu et al. 2012). In contrast, only one sigma class GST gene is found in the *D. melanogaster* genome (Tu and Akgul 2005) as well as the *A. gambiae* mosquito, with two distinct transcripts arising from alternative splicing (Ding et al. 2003).

Phylogenetic analysis was also performed to investigate the evolutionary position of AccGSTS1 and its homologues in insects (Fig. 1a). As expected, the tree classified AccGSTS1 alongside other sigma class GSTs. Alignment of AccGSTS1 with its homologues in *A. mellifera*, *B. mori*, *D. melanogaster*, *A. gambiae*, *Locusta migratoria*, and *Nasonia vitripennis*, demonstrated 72.86–98 % similarity. Residues at the N-terminal domains of these GSTs, which contribute to the binding of GSH, were shown to be conserved (Fig. 1b), including a Tyr⁸ that is critical for stabilization of GSH (Blanchette et al. 2007). On the contrary, residues at the C-terminal domain, where the substrate binding site (H-site) is located, were highly diverse. The 3D molecular structure of AccGSTS1 was predicted by SWISS-MODEL using 1m0uA (1.75 Å) as the template (Fig. 1c). These two proteins share 37.811 % sequence identity, and the model showed that AccGSTS1 possesses the typical cytosolic GST structure, including a conserved N-terminal domain with a $\beta\alpha\beta\alpha\beta\alpha$ motif and a C-terminal domain composed entirely of α -helices.

Genomic structure and copy number of AccGSTS1

To elucidate the genomic DNA structure of *AccGSTS1*, the genomic sequence was amplified using genomic DNA as the template for PCR amplification. The full-length *AccGSTS1* genomic sequence (GenBank accession number HQ828082) is 1,865 bp. The positions and sizes of the introns were noted by aligning the cDNA sequences of AccGSTS1 with the genomic DNA sequence. There are a total of three introns, each of which is within the ORF. The nucleotide sequences at the splice junctions are consistent with the canonical GT–AG rule. Next, the genomic sequence of *AccGSTS1* was aligned with other *GSTS1* sequences; interestingly, although their intron numbers and lengths are different, *AccGSTS1*, *BmGSTS1*, and *AgGSTS1* share a conserved intron positioned at the 44th codon (Fig. 2). Likewise, previous reports have demonstrated that almost half of the GSTs in the silkworm (Yu et al. 2008) and mosquito (Ding et al. 2003) share a conserved position at approximately the 50th codon from the 5' end of the gene. Intron positions have been successfully used to delineate the deep phylogenetic relationships (Rogozin et al. 2005). Thus, although the putative AccGSTS1 shows a greater identity to AmGSTS1 than BmGSTS1 or AgGSTS1, AccGSTS1 may have a closer relationship with BmGSTS1 and AgGSTS1. The elucidation of the genomic sequence of AccGSTS1 in

Fig. 1 Molecular characterization of AccGSTS1. **a** Phylogenetic relationship between AccGSTS1 and GSTs from *A. cerana cerana*, *A. mellifera*, *N. vitripennis*, *B. mori*, *A. gambiae*, and *D. melanogaster*, in addition to GSTs from other insects. Species represented: *Cr*, *Chironomus riparius*; *Ss*, *Sus scrofa*; *Hs*, *Harpegnathos saltator*; *Ac*, *Anopheles cracens*; *Ad*, *Anopheles dirus*; and *Lm*, *Locusta migratoria*. The tree was constructed using NJ-based bootstrapped phylogeny in MEGA 4 with 1,000 bootstrap replicates; values are indicated at each node. AccGSTS1 is boxed. The GenBank accession numbers of protein sequences are indicated in parentheses. **b** Sequence alignment of AccGSTS1 with known homologs. GSTs from *A. mellifera*

(NP_001153742), *N. vitripennis* (NP_001165918), *B. mori* (NP_001037077), *A. gambiae* (XP_311546), *L. migratoria* (AEB91973), and *D. melanogaster* (NP_725653) were used. Identical residues are shaded black, while 75 % conserved residues are shown in gray. The catalytic residue Tyr is marked with triangle. The GSH-binding sites and substrate-binding sites are highlighted with daggers and section symbols, respectively. **c** The tertiary structure of AccGSTS1. The N-terminal domain (ββαββ motif) containing the G-site and the C-terminal domain (all α-helical motifs) harboring the H-site are shown. The conserved Tyr at position 8 responsible for the stabilization of GSH is indicated

this study establishes a platform for deepening our understanding of the organization of the *A. cerana cerana* GST superfamily.

We next performed qPCR to investigate the copy number of *AccGSTS1*. *AccGSTZ1*, which we previously demonstrated to be a single-copy gene by Southern blot (Yan et al. 2012), was used as an internal standard. The correlation coefficients of *AccGSTZ1* and *AccGSTS1* were rather good, i.e., 0.999 (Supplementary Figure 1), and these results demonstrate that *AccGSTS1* is present as a single copy in the *A. cerana cerana* genome (Table 3).

Tissue and stage-specific expression profiles of AccGSTS1

The distribution of *AccGSTS1* was analyzed by qPCR using multiple tissues from adult worker bees, including the brain, fat body, epidermis, muscle, and midgut. As shown in Fig. 3a, the mRNA level of *AccGSTS1* was most robust in the fat body, and the expression was appreciable in the midgut, but the transcripts in the brain were virtually undetectable, suggesting tissue-specific expression patterns. In insects, the fat body and midgut are the central metabolic organs that play important roles in detoxification/degradation of xenobiotics and protection from

Fig. 2 Schematic representation of intron positions of sigma class GST genes. Lengths of genomic DNA sequences from *A. cerana cerana* (HQ828082), *A. mellifera* (NW_001254614.1), *B. mori* (DQ862466), *A. gambiae* (NT_078267.5), and *D. melanogaster*

(NT_033778.3) are indicated according to the scale below. *Light gray* and *gray* are used to highlight the exons and introns, respectively. The initiation and termination codons are indicated by inverted triangles and asterisks, respectively

oxidative stress (Arrese and Soulages 2010; Enayati et al. 2005; Sawicki et al. 2003). In addition, there is evidence that insect GSTs can detoxify many plant allelochemicals and synthetic insecticides (Li et al. 2007). Thus, the higher mRNA levels of *AccGSTS1* in these two tissues suggest a potential functional role in protection against the toxicity of xenobiotics present in the diet of honey bees. GST genes have also been reported to be widely expressed in the fat body and midgut of other insect species (Chintapalli et al. 2007; Krishnan and Kodrik 2006). In *D. melanogaster*, 28 out of 38 (73.7 %) GST genes, belonging to a total of 5 classes, are expressed in the midgut of third instar larvae (Li et al. 2008), and in *B. mori*, the percentage is 73.9 % (Yu et al. 2008). GSTs have also been found in large concentrations in other tissues. For example, DmGSTS1-1 was thought to be anchored to the flight muscle of *D. melanogaster* by the hydrophobic/acidic N-terminal extension and was demonstrated to be instrumental in protecting this highly aerobic tissue from by-products of oxidative stress (Singh et al. 2001). Although this N-terminal extension on the sigma class GST also occurs in some other Muscamorphan insects, such as *Musca domestica*, *Glossina morsitans*, and *Mayetola destructor*, anchoring of this GST to the flight muscle is not universal. Absence of such GST proteins in other insects including *A. cerana cerana* suggests that there may be quite substantial differences in the biochemistry of flight muscle between different insect groups (Flanagan and Smythe 2011).

To determine the regulation of *AccGSTS1* at different developmental stages, the expression of *AccGSTS1* in larvae, pupae, and adults was measured by qPCR. Larvae and

pupae worker honey bees were generally classified according to age, eye color, and shape. As for the adults, we chose the 1-day-old bees and 2-week-old foragers mainly considering the role of *AccGSTS1* in oxidative stress. Only foragers have the chance to go outside of the hive and exposed to external environment and therefore challenged by excessive ROS and oxidative stress. Both of 1-day-old bees and 2-week-old hive bees are alive in the comb and suffered less oxidative damage than foragers. Using bees at the stage of 1-day-old bees and 2-week-old foragers can not only determined the expression of *AccGSTS1* at different development stages but also considered the factors of oxidative stress. Moreover, behavior may affect the expression of *AccGSTS1* in 2-week-old foragers and hive bees; further studies could be done to verify this possibility. The results suggested the developmental regulation of *AccGSTS1* with the highest mRNA levels in adults (Fig. 3b), the stage at which the bees have the chance to leave the hive and forage for nectar and pollen for the remainder of their lives (Ament et al. 2008). The transcripts remained unchanged from the last-instar larvae to dark eye pupae, indicating that *AccGSTS1* may be constitutively expressed in the pupae. Maximum expression of *AccGSTS1* was shown to occur during a period in the lifecycle of the bees when they are exposed to the external environment. It is during this time that they encounter various stresses and are therefore more susceptible to toxic chemicals and oxidative damage. In *D. melanogaster*, the sole sigma class GST plays a dominant protective role against oxidative damage (Singh et al. 2001; Alias and

Table 3 Estimation of copy number of *AccGSTS1* gene in *Apis cerana cerana*

Experiments	AccGSTS1		AccGSTZ1		AccGSTS1/ AccGSTZ1	Copy number
	Ct values	Calculation results	Ct values	Calculation results		
1	25.27	−3.97	25.01	−4.19	0.95	1
2	27.09	−4.56	26.52	−4.68	0.97	1
3	29	−5.18	28.5	−5.34	0.97	1

Fig. 3 Transcriptional patterns of *AccGSTS1* in different tissues and during different stages of development. **a** Distribution of *AccGSTS1* in the brain (BR), fat body (FB), epidermis (EP), muscle (MS), and midgut (MG). **b** The mRNA transcripts of *AccGSTS1* at the following developmental stages: larvae (L3–L6 instars), pupae (Pw white eyes, Pp pink eyes, and Pd dark eyes) and adults (A1 1-day postemergence and A14 14-day post-emergence adults). The mRNA expression of *AccGSTS1* was quantified and normalized to that of the *A. cerana cerana* β -actin gene. Vertical bars represent the mean $\pm$ SD ($n=3$). Different letters above the bar indicate significant differences, according to $P<0.01$ in SAS

Clark 2007). Taken together, the high expression of *AccGSTS1* at this stage indicates that it may play important roles in detoxification as well as antioxidant defense.

Induction of *AccGSTS1* after exposure to various environmental stresses

GSTs are known to be an important component of the oxidative stress response. Previous studies have indicated that environmental conditions such as temperature, heavy metals, salinity, pesticides, and UV radiation can induce oxidative stress (Lushchak 2011; Kottuparambilla et al. 2012). To elucidate the potential involvement of *AccGSTS1* in antioxidant defense, transcripts of *AccGSTS1* under cold (4 °C), heat (43 °C), H_2O_2 , $HgCl_2$, and insecticide (phoxim, cyhalothrin, and acaricide) exposure were examined by qPCR (Fig. 4). *AccGSTS1* expression was upregulated under all treatments examined, although the mRNA levels were increased by different degrees. Alterations to GST expression induced by various factors have been extensively reported (Meng et al. 2009; Nair and Choi 2011; Umasuthan et al. 2012; Yang et al. 2012), and GSTs are

Fig. 4 Expression patterns of *AccGSTS1* under various abiotic stresses. **a** The mRNA levels of *AccGSTS1* upon exposure to cold (4 °C), heat (43 °C), UV radiation, and H_2O_2 . Samples were collected at 0, 1, 2, 3, and 4 h after different treatments. **b** The expression level of *AccGSTS1* after bees were fed with a heavy metal ($HgCl_2$) and insecticides (phoxim, cyhalothrin, and acaricide) for 0, 0.5, 1, 1.5, and 2 h. The mRNA expression of *AccGSTS1* was quantified and normalized to that of the *A. cerana cerana* β -actin gene. Vertical bars represent the mean $\pm$ SD ($n=3$). Different letters above the bar indicate significant differences, according to $P<0.01$ in SAS

known to act as detoxification agents against the chemotoxicity of xenobiotics. Elevated levels of GST are also correlated with resistance to insecticides (Ranson et al. 2001; Vontas et al. 2001). Moreover, in *Nilaparvata lugens*, GST levels are elevated primarily to protect tissues by conferring resistance against oxidative damage (Vontas et al. 2001). Thus, the high expression of *AccGSTS1* may present a defense mechanism against increased levels of oxidative stress caused by different environmental challenges. The induction of GST has been proposed to represent an evolutionarily conserved cellular response to oxidative stress (Hayes et al. 2005), and individual GSTs present in different insect species or different subclasses within the same species are regulated independently in response to various stressors. Among the six classes of GSTs, sigma, as well as delta and epsilon GSTs, has been shown to play key roles in antioxidant defense in various organisms (Singh et al. 2001; Hayes et al. 2005; Oakley 2005; Frova 2006; Blanchette et al. 2007). Taken together, we hypothesize that upregulated *AccGSTS1* expression may be associated with antioxidant tolerance in honey bees.

Transgenic worms expressing a *Caenorhabditis elegans* *GSTO-1::GFP* fusion protein under the control of the

GSTO-1 promoter experienced transcriptional upregulation of *GSTO-1* under pro-oxidant stress, which subsequently led to an increase in stress resistance in the worms (Burmeister et al. 2008). In particular, the overexpression of *C. elegans* GSTP2-2 in transgenic worms was found not only to lead to resistance to paraquat, heat shock, UV radiation, and hydrogen peroxide but also to an increased median lifespan (Ayyadevara et al. 2005). Therefore, an increase in *AccGSTS1* expression under various abiotic stresses may protect honey bees from ROS damage to ensure survival and may represent an adaptive response against oxidative stress.

Bacterial expression and purification of AccGSTS1

AccGSTS1 was overexpressed as a fusion protein with a cleavable N-terminal His-tag in *E. coli* BL21 (DE3). SDS-PAGE analysis revealed that this AccGSTS1 fusion protein was expressed in a soluble form after induction with IPTG (Fig. 5). The recombinant protein had a molecular size of approximately 23 kDa, which is in agreement with its predicted value. This recombinant protein was further purified using an Ni-nitrilotriacetic acid spin column. Finally, 4.8 mg of highly purified AccGSTS1 from 250 mL of LB medium was obtained. In previous studies, recombinant Sigma class GSTs from the fall webworm (Yamamoto et al. 2007), silkworm (Yamamoto et al. 2006), and earthworm (LaCourse et al. 2009) exhibited specific activities for CDNB ranging between 1.9 and 4.4 $\mu\text{mol min}^{-1}\text{mg}^{-1}$. In the present study, the specific activity of the final product toward CDNB was 2.1 $\mu\text{mol min}^{-1}\text{mg}^{-1}$, revealing that active AccGSTS1 was successfully expressed in *E. coli* cells.

Fig. 5 Overexpression and purification of recombinant AccGSTS1 fusion protein in *E. coli* BL21 (DE3). Lanes M protein marker; U and I un-induced and IPTG-induced cellular extract, respectively; P purified AccGSTS1 protein

Fig. 6 Effect of AccGSTS1 on the removal of H_2O_2 . Peroxidase activity of AccGSTS1 in different concentrations and incubation times. Abscissa indicates different concentration of recombinant protein (10, 20, 50, and 100 $\mu\text{g/ml}$) added in the reaction mixture. White, light gray, dark gray, and black represent incubation times (0, 2, 5, and 10 min, respectively). Values shown represent the mean (SD) of three experiments

Biochemical characterization of AccGSTS1

As sigma class proteins have been shown to be involved in lipid peroxidation in *Drosophila* (Agianian et al. 2003), we next determined the antioxidant properties of AccGSTS1 by measuring the removal of H_2O_2 in a reaction mixture with DTT as an electron donor (Suttiprapa et al. 2008). As shown in Fig. 6, the rate of H_2O_2 destruction was gradually increased with increasing concentrations of recombinant protein and incubation time, suggesting that AccGSTS1 can catalyze the removal of H_2O_2 in a concentration- and time-dependent manner. To further evaluate the capacity of AccGSTS1 to protect DNA from oxidative damage, we employed a thiol-dependent MFO system, in which hydroxyl radicals are produced and convert supercoiled plasmid DNA into its nicked form (lane 3) (Fig. 7). The purified AccGSTS1 was found to prevent supercoiled pUC19 from degrading in a dose-dependent manner (lanes 4–9). Protection against DNA cleavage in the thiol-MFO system was due to the removal of activated oxygen species (Lim et al. 1993), suggesting that AccGSTS1 may be involved in the protection mechanism under oxidative stress. In addition, DNA repair capacity has been shown to correlate with increased stress resistance and thus may lead to a longer lifespan

Fig. 7 Ability of AccGSTS1 to protect against DNA damage in the MFO system. Lane 0 pUC19 plasmid DNA only; lane 1 pUC19 plasmid DNA + FeCl_3 ; lane 2 pUC19 plasmid DNA + FeCl_3 + dithiothreitol (DTT); lane 3 pUC19 plasmid DNA + FeCl_3 + BSA; lanes 4–9 pUC19 plasmid DNA + FeCl_3 + DTT + purified AccGSTS1 (5, 10, 50, 100, 150, and 200 mg/ml, respectively). SF supercoiled form, NF nicked form

(Hyun et al. 2008). Therefore, AccGSTS1 may also be associated with the lifespan of the honey bee. GST peroxidase activity is of particular importance to insects because they do not possess any selenium-dependent glutathione peroxidase activity and therefore depend on

GSTs for the reduction of organic hydroperoxides (Singh et al. 2001). The observation that AccGSTS1 could remove H_2O_2 and protect DNA indicates that AccGSTS1 may play an important role in the survival of insects under oxidative stress.

To provide direct evidence that AccGSTS1 is responsible for antioxidant defense, a disc diffusion assay was performed. *E. coli* cells overexpressing AccGSTS1 were exposed to paraquat, *t*-butylhydroperoxide and cumene hydroperoxide, which are known oxidative stress inducers (Burmeister et al. 2008). Following overnight exposure, the inhibition zones of the bacteria expressing AccGSTS1 were found to be much smaller than those of cells transfected with the vector only (Fig. 8a). The halo size reductions were 18 % for paraquat, 19 % for *t*-butylhydroperoxide, and 41 % for cumene hydroperoxide (Fig. 8b). Earlier studies have demonstrated that paraquat treatment results in oxidative stress and induces the expression of several classes of GST genes in *C. riparius* (Nair and Choi 2011). Similarly, cumene hydroperoxide and *t*-butylhydroperoxide are also widely used as model substances for studying the mechanism of cell injury resulting from oxidative stress (Sawicki et al. 2003; Tang and Tu 1994). In the present study, paraquat, *t*-butylhydroperoxide and cumene hydroperoxide adsorbed to the filter disc may have induced oxidative stress in *E. coli* cells. The decrease in the size of the killing zones for cells over-expressing AccGSTS1 provides further evidence that AccGSTS1 can serve as an effective antioxidant enzyme that protects cells from oxidative stress.

Conclusions

In the present study, we identified and characterized a sigma class GST from *A. cerana cerana*. This enzyme (AccGSTS1) is represented by a single locus in the genome and possesses the conserved functional domains of the GST superfamily. The temporal expression levels of *AccGSTS1* in response to various environmental stresses indicate that *AccGSTS1* may play a biological role in oxidative stress-related defense mechanisms. Its induction under $HgCl_2$, phoxim, cyhalothrin, and acaricide exposure may result in the enhanced tolerance of *A. cerana cerana* to other insecticides and xenobiotics. Recombinant AccGSTS1 was shown to remove H_2O_2 and protect DNA from oxidative damage, and a disc diffusion assay demonstrated a protective role upon exposure to oxidative stress. Taking into account these observations, our results suggest that AccGSTS1 may be involved in detoxification mechanisms and may be an important antioxidant enzyme against oxidative stress. In conclusion, these findings may be useful for studying the comparative molecular functions of different GST classes across various insect lineages.

Fig. 8 Antioxidant activity of AccGSTS1 according to a disc diffusion assay. **a** Approximately 5×10^8 *E. coli* cells overexpressing AccGSTS1 were flooded on the LB agar plates. Paper discs, which adsorbed paraquat, cumene hydroperoxide, and *t*-butylhydroperoxide in concentration gradients, were placed on the agar plates seeded with bacteria. The plates were incubated for 24 h at 37 °C. The labels 0, 1, 2, 3, 4, and 5 on filter discs represent the different concentrations of paraquat (0, 50, 100, 150, 200, and 250 μ M, respectively), cumene hydroperoxide (0, 50, 100, 150, 200, and 250 mM, respectively), and *t*-butylhydroperoxide (0, 25, 50, 100, 200, and 300 mM, respectively). Bacteria transfected with pET-30a (+) (vector only) were used as controls. The halo diameter of the inhibition zones is shown in **b**

Acknowledgments This work was funded by the China Agriculture Research System (no. CARS-45), Agro-scientific Research in the Public Interest (number 200903006) and the National Natural Science Foundation (number 31172275) in China.

References

- Agianian B, Tucker PA, Schouten A, Leonard K, Bullard B, Gros P (2003) Structure of a *Drosophila* sigma class glutathione S-transferase reveals a novel active site topography suited for lipid peroxidation products. *J Mol Biol* 326:151–165
- Alias Z, Clark AG (2007) Studies on the glutathione S-transferase proteome of adult *Drosophila melanogaster*: responsiveness to chemical challenge. *Proteomics* 7:3618–3628
- Ament SA, Corona M, Pollock HS, Robinson GE (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc Natl Acad Sci USA* 105:4226–4231
- Arrese EL, Soulages JL (2010) Insect fat body: energy, metabolism, and regulation. *Ann Rev Entomol* 55:207–225
- Ayyadevara S, Engle MR, Singh SP, Dandapat A, Lichti CF, Benes H, Shmookler Reis RJ, Liebau E, Zimniak P (2005) Lifespan and stress resistance of *Caenorhabditis elegans* are increased by expression of glutathione transferases capable of metabolizing the lipid peroxidation product 4-hydroxynonenal. *Aging Cell* 4:257–271
- Bairoch A, Bucher P, Hofmann K (1997) The PROSITE database, its status in 1997. *Nucleic Acids Res* 25:217–221
- Blanchette B, Feng X, Singh BR (2007) Marine glutathione S-transferases. *Mar Biotechnol* 9:513–542
- Burmeister C, Luërsen K, Heinick A, Hussein A, Domagalski M, Walter RD, Liebau E (2008) Oxidative stress in *Caenorhabditis elegans*: protective effects of the Omega class glutathione transferase (*GSTO-1*). *FASEB J* 22:343–354
- Che-Mendoza A, Penilla RP, Rodríguez DA (2009) Insecticide resistance and glutathione S-transferases in mosquitoes: a review. *Afr J Biotechnol* 8:1386–1397
- Chen L, Hall PR, Zhou XE, Ranson H, Hemingway J, Meehan EJ (2003) Structure of an insect delta-class glutathione S-transferase from a DDT-resistant strain of the malaria vector, *Anopheles gambiae*. *Acta Crystallogr D* 59:2211–2217
- Chintapalli VR, Wang J, Dow JAT (2007) Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat Genet* 39:715–720
- Corona M, Robinson GE (2006) Genes of the antioxidant system of the honey bee: annotation and phylogeny. *Insect Mol Biol* 15:687–701
- Ding Y, Ortellì F, Rossiter LC, Hemingway J, Ranson H (2003) The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genom* 4:35
- Ding Y, Hawkes N, Meredith J, Eggleston P, Hemingway J, Ranson H (2005) Characterisation of the promoters of Epsilon glutathione transferases in the mosquito *Anopheles gambiae* and their response to oxidative stress. *Biochem J* 387:879–888
- Durou C, Poirier L, Amiard JC, Budzinski H, Gnassia-Barelli M, Lemenach K, Peluhet L, Mouneyrac C, Romeo M, Amiard-Triquet C (2007) Biomonitoring in a clean and a multi-contaminated estuary based on biomarkers and chemical analyses in the endobenthic worm *Nereis diversicolor*. *Environ Pollut* 148:445–458
- Enayati AA, Ranson H, Hemingway J (2005) Insect glutathione transferases and insecticide resistance. *Insect Mol Biol* 14:3–8
- Flanagan JU, Smythe ML (2011) Sigma-class glutathione transferases. *Drug Metab Rev* 43:194–214
- Frova C (2006) Glutathione transferases in the genomics era: new insights and perspectives. *Biomol Eng* 23:149–169
- Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249:7130–7139
- Hayes JD, Pulford DJ (1995) The glutathione S-transferase supergene family. Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* 30:445–600
- Hayes JD, Flanagan JU, Jowsey IR (2005) Glutathione transferases. *Annu Rev Pharmacol Toxicol* 45:51–88
- Hyun M, Lee J, Lee K, May A, Bohr VA, Ahn B (2008) Longevity and resistance to stress correlate with DNA repair capacity in *Caenorhabditis elegans*. *Nucleic Acids Res* 36:1380–1389
- Kampkotter A, Volkmann TE, de Castro SH, Leiers B, Klotz LO, Johnson TE, Link CD, Henkle-Duhrsen K (2003) Functional analysis of the glutathione S-transferase 3 from *Onchocerca volvulus* (*Ov*-GST-3): a parasite GST confers increased resistance to oxidative stress in *Caenorhabditis elegans*. *J Mol Biol* 325:25–37
- Kostaropoulos I, Papadopoulos AI, Metaxakis A, Boukouvala E, Papadopolou-Mourkidou E (2001) Glutathione S-transferase in the defence against pyrethroids in insects. *Insect Biochem Mol Biol* 31:313–319
- Kottuparambala S, Shinb W, Brownc MT, Han T (2012) UV-B affects photosynthesis, ROS production and motility of the freshwater flagellate, *Euglena agilis* Carter. *Aquat Toxicol* 122–123:206–213
- Krishnan N, Kodrik D (2006) Antioxidant enzymes in *Spodoptera littoralis* (Boisduval): are they enhanced to protect gut tissues during oxidative stress? *J Insect Physiol* 52:11–20
- LaCourse EJ, Hernandez-Viadel M, Jefferies JR, Svendsen C, Spurgeon DJ, Barrett J, Morgan AJ, Kille P, Brophy PM (2009) Glutathione transferase (GST) as a candidate molecular-based biomarker for soil toxin exposure in the earthworm *Lumbricus rubellus*. *Environ Pollut* 157:2459–2469
- Li X, Schuler MA, Berenbaum MR (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Ann Rev Entomol* 52:231–253
- Li HM, Buczkowski G, Mittapalli O, Xie J, Wu J, Westerman R, Schemerhorn BJ, Murdock LL, Pittendrigh BR (2008) Transcriptomic profiles of *Drosophila melanogaster* third instar larval midgut and responses to oxidative stress. *Insect Mol Biol* 17:325–339
- Lim YS, Cha MK, Uhm TB, Park JW, Kim K, Kim IH (1993) Removals of hydrogen peroxide and hydroxyl radical by thiol-specific antioxidant protein as a possible role in vivo. *Biochem Biophys Res Commun* 192:273–280
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408
- Low WY, Ng HL, Morton CJ, Parker MW, Batterham P, Robin C (2007) Molecular evolution of glutathione S-transferases in the genus *Drosophila*. *Genetics* 177:1363–1375
- Lumjuan N, Mccarroll L, Prapanthadara LA, Hemingway J, Ranson H (2005) Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector. *Aedes aegypti*. *Insect Biochem Mol Biol* 35:861–871
- Lumjuan N, Rajatileka S, Changsom D, Wicheer J, Leelapat P, Prapanthadara L, Somboon P, Lycett G, Ranson H (2011) The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. *Insect Biochem Mol Biol* 41:203–209
- Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* 101:13–30
- Mason G, Provero P, Vaira AM, Accotto GP (2002) Estimating the number of integrations in transformed plants by quantitative real-time PCR. *BMC Biotechnol* 2:20

- Meng JY, Zhang CY, Zhu F, Wang XP, Lei CL (2009) Ultraviolet light-induced oxidative stress: effects on antioxidant response of *Helicoverpa armigera* adults. *J Insect Physiol* 55:588–592
- Nair PMG, Choi J (2011) Identification characterization and expression profiles of *Chironomus riparius* glutathione S-transferase (GST) genes in response to cadmium and silver nanoparticles exposure. *Aquat Toxicol* 101:550–560
- Nair PMG, Park SY, Choi J (2011) Expression of catalase and glutathione S-transferase genes in *Chironomus riparius* on exposure to cadmium and nonylphenol. *Com Biochem Physiol C* 154:399–408
- Oakley AJ (2005) Glutathione transferases: new functions. *Curr Opin Struct Biol* 15:716–723
- Ranson H, Jensen B, Wang X, Prapanthadara L, Hemingway J, Collins FH (2000) Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Mol Biol* 9:499–507
- Ranson H, Rossiter L, Orтели F, Jensen B, Wang X, Roth CW (2001) Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochem J* 359:295–304
- Rogers ME, Jani MK, Vogt RG (1999) An olfactory-specific glutathione-S-transferase in the sphinx moth *Manduca sexta*. *J Exp Biol* 202:1625–1637
- Rogozin IB, Sverdlov AV, Babenko VN, Koonin EV (2005) Analysis of evolution of exon–intron structure of eukaryotic genes. *Brief Bioinform* 6:118–134
- Sawicki R, Singh SP, Mondal AK, Benes H, Zimniak P (2003) Cloning, expression and biochemical characterization of one Epsilon class (GST-3) and ten Delta-class (GST-1) glutathione S-transferases from *Drosophila melanogaster*, and identification of additional nine members of the Epsilon class. *Biochem J* 370:661–669
- Singh SP, Coronella JA, Benes H, Cochrane BJ, Zimniak P (2001) Catalytic function of *Drosophila melanogaster* glutathione S-transferase DmGSTS1-1 (GST-2) in conjugation of lipid peroxidation end products. *Eur J Biochem* 268:2912–2923
- Suttiaprapa S, Loukas A, Laha T, Wongkham S, Kaewkes S, Gaze S, Brindley PJ, Srija B (2008) Characterization of the antioxidant enzyme, thioredoxin peroxidase, from the carcinogenic human liver fluke, *Opisthorchis viverrini*. *Mol Biochem Parasitol* 160:116–122
- Tang AH, Tu CP (1994) Biochemical characterization of *Drosophila* glutathione S-transferases D1 and D21. *J Biol Chem* 269:27876–27884
- Tu CP, Akgul B (2005) *Drosophila* glutathione S-transferases. *Methods Enzymol* 401:204–226
- Umasuthan N, Revathy KS, Lee Y, Whang I, Choi CY, Lee J (2012) A novel molluscan sigma-like glutathione S-transferase from Manila clam, *Ruditapes philippinarum*: Cloning, characterization and transcriptional profiling. *Com Biochem Physiol C* 155:539–550
- Vontas JG, Small GJ, Hemingway J (2001) Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem J* 357:65–72
- Xu P, Shi M, Chen XX (2009) Antimicrobial peptide evolution in the Asiatic honey bee *Apis cerana*. *PLoS One* 4:e4239
- Yamamoto K, Zhang PB, Banno Y, Fujii H (2006) Identification of a sigma-class glutathione-S-transferase from the silkworm, *Bombyx mori*. *J Appl Entomol* 130:515–522
- Yamamoto K, Fujii H, Aso Y, Banno Y, Koga K (2007) Expression and characterization of a sigma-class glutathione S-transferase of the fall webworm, *Hyphantria cunea*. *Biosci Biotechnol Biochem* 71:553–560
- Yan HR, Meng F, Jia HH, Guo XQ, Xu BH (2012) The identification and oxidative stress response of a zeta class glutathione S-transferase (GSTZ1) gene from *Apis cerana cerana*. *J Insect Physiol* 58:782–791
- Yang ML, Zhang JZ, Zhu KY, Xuan T, Liu XJ, Guo YP, Ma EB (2009) Mechanisms of organophosphate resistance in a field population of oriental migratory locust, *Locusta migratoria manilensis* (Meyen). *Arch Insect Biochem Physiol* 71:3–15
- Yang J, Wei XM, Xu J, Yang DL, Liu XQ, Yang JM, Fang JH, Hu XK (2012) A sigma-class glutathione S-transferase from *Solen grandis* that responded to microorganism glycan and organic contaminants. *Fish Shellfish Immunol* 32:1198–1204
- Yu QY, Lu C, Li B, Fang SM, Zuo WD, Dai FY, Zhang Z, Xiang ZH (2008) Identification, genomic organization and expression pattern of glutathione S-transferase in the silkworm, *Bombyx mori*. *Insect Biochem Mol Biol* 38:1158–1164
- Yu XL, Sun RJ, Yan HR, Guo XQ, Xu BH (2012) Characterization of a sigma class glutathione S-transferase gene in the larvae of the honeybee (*Apis cerana cerana*) on exposure to mercury. *Comp Biochem Physiol B* 161:356–364