Eur. J. Entomol. **105**: 567–574, 2008
http://www.eje.cz/scripts/viewabstract.php?abstract=1370
ISSN 1210-5759 (print), 1802-8829 (online)

Glutathione S-transferases from the larval gut of the silkworm *Bombyx mori*: cDNA cloning, gene structure, expression and distribution

ZHONG ZHENG GUI[1, 2], BO YEON KIM[1], KWANG SIK LEE[1], YA DONG WEI[1,2], XIJIE GUO[2], HUNG DAE SOHN[1] and BYUNG RAE JIN[1]*

[1]College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea
[2]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China

Key words. Baculovirus expression vector, *Bombyx mori*, cDNA cloning, enzyme, gene structure, glutathione S-transferase, silkworm

Abstract. Two glutathione S-transferase (GST) cDNAs, GSTD2 and GSTS2, were cloned from the silkworm *Bombyx mori*. The *B. mori* GSTD2 (*BmGSTD2*) gene spans 4371 bp and consists of four introns and five exons that encode 222 amino acid residues. The deduced amino acid sequence of *BmGSTD2* showed 58% protein sequence identity to the Delta-class GST of *Maduca sexta*. The *B. mori* GSTS2 (*BmGSTS2*) gene spans 3470 bp and consists of three introns and four exons that encode 206 amino acid residues. The deduced amino acid sequence of *BmGSTS2* revealed 67%, 63%, and 61% protein sequence identities to the Sigma-class GSTs from *B. mori*, *Platynota idaeusalis*, and *M. sexta*, respectively. The *BmGSTD2* and *BmGSTS2* cDNAs were expressed as 25 kDa and 23 kDa polypeptides, respectively, in baculovirus-infected insect Sf9 cells. Northern blot and Western blot analyses showed that BmGSTD2 and BmGSTS2 were specifically expressed in three gut regions, indicating that the gut is the prime site for BmGSTD2 and BmGSTS2 synthesis in *B. mori* larvae.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are detoxification enzymes that catalyze the conjugation of electrophilic compounds with the thiol group of reduced glutathione (GSH). This modification generally increases the solubility of the resultant products in water and therefore renders them more excretable than non-GSH conjugated substrates. GSTs are implicated in the detoxification of both endogenous and xenobiotic compounds and are involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress (reviewed by Enayati et al., 2005). Mammalian GSTs have been classified into seven classes: Alpha, Mu, Pi, Theta, Sigma, Zeta, and Omega (Mannervik et al., 2005). In line with the mammalian GST classification system, insect GSTs have been grouped into six classes: Delta, Epsilon, Omega, Theta, Sigma, and Zeta (Chelvanayagam et al., 2001; Ranson et al., 2002; Claudianos et al., 2006).

Insect GSTs are of particular interest because of their role in insecticide resistance. An increase in the activity of these enzymes is associated with resistance to all of the major classes of insecticides (Fournier et al., 1992; Prapanthadara et al., 1993; Huang et al., 1998; Ranson et al., 2001; Vontas et al., 2001; Ortelli et al., 2003; Enayati et al., 2005; Lumjuan et al., 2005). Due to the important role of GST, GST genes have been cloned from several insect species (Snyder et al., 1995; Arruda et al., 1997; Ranson et al., 1997; Ding et al., 2003; Valles et al., 2003; Claudianos et al., 2006; Yamamoto et al., 2005, 2006, 2007).

The silkworm, *B. mori*, is an economically important animal that has been used for centuries for silk production and recently also for the production of recombinant proteins using a baculovirus expression system (Lee et al., 2006, 2007). In spite of the particular interest in insect GSTs and the economic importance of silkworm, there is relatively little research on the role and gene structure of silkworm GSTs. Recently, studies have described the cloning of silkworm Delta-class and Sigma-class GST cDNAs (Yamamoto et al., 2005, 2006). This paper describes the cloning of two novel genes encoding a Delta-class GST (BmGSTD2) and a Sigma-class GST (BmGSTS2) from *B. mori*. We cloned and sequenced the cDNAs and genomic DNAs of two novel GSTs of *B. mori* larvae, and expressed the recombinant GSTs in baculovirus-infected insect cells. The distributions of BmGSTD2 and BmGSTS2 were determined by the transcriptional and translational levels of the GSTs in *B. mori* larvae.

MATERIAL AND METHODS

Animals

The *Bombyx mori* larvae used in this study were F_1 hybrid Baekok-Jam supplied by the Department of Agricultural Biology, The National Institute of Agricultural Science and Technology, Korea. Silkworms were reared on fresh mulberry leaves at 25°C, 65 ± 5% relative humidity and a 12L : 12D photoperiod.

* Corresponding author; e-mail: brjin@dau.ac.kr

cDNA library screening, nucleotide sequencing and data analysis

The clone containing the cDNA insert was selected from expressed sequence tags (ESTs), which were generated from a cDNA library constructed using whole bodies of *B. mori* larvae (Kim et al., 2003). The plasmid DNA was extracted using a Wizard mini-preparation kit (Promega, Madison, WI, USA) and sequenced using an ABI 310 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). MacVector (Version 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the amino acid sequences of the GSTs. Phylogenetic analysis was performed upon the GenBank registered GST amino acid sequences using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2000).

Genomic DNA isolation and genomic PCR

Genomic DNA was extracted from the alimentary canal of larvae of *B. mori* using a Wizard™ Genomic DNA Purification Kit, according to the manufacturer's instructions (Promega). The primers used for amplification of the genomic DNAs encoding BmGSTD2 and BmGSTS2 were 5'-ATGGTGCTAACACTACAAGATG-3' for the translational start sequence region of BmGSTD2, 5'-TTATTCTTTTCCACG ACTCAGG-3' for the 3' non-coding region of BmGSTD2, 5'-ATGCCTAAGGTTGTGTACCATTACTTCGC-3' for the translational start sequence region of BmGSTS2 and 5'-TTAGAATTCAAATTGGTATGGCCTTCCC-3' for the 3' non-coding region of BmGSTS2, based on the *BmGSTD2* and *BmGSTS2* cDNAs cloned in this study. PCR was done at 94°C for 2 min, 35 cycles of amplification (94°C for 1 min; 55°C for 1 min; 72°C for 1 min) and 72°C for 10 min. The resulting fragment was analyzed by electrophoresis on a 1.0% agarose gel. The PCR products for sequencing were cloned into the pGem-T vector (Promega) and the constructs were then transformed into *Escherichia coli* TOP10F' cells (Invitrogen, Carlsbad, CA, USA). The nucleotide sequence was determined using a BigDyeTerminator cycle sequencing kit and an automated DNA sequencer, as described above.

RNA isolation and northern blot analysis

The fifth instar *B. mori* larvae were dissected on ice under a stereo-microscope (Zeiss, Jena, Germany). Individual samples of the alimentary canal, fat body, haemocytes, silk gland, and epidermis were collected and washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). For northern blot analysis of the *B. mori* digestive tract, dissection of the foregut and hindgut was minimized to avoid contamination of the adjacent midgut tissues. Total RNA was isolated from the gut (foregut, midgut and hindgut), fat body, haemocytes, silk gland and epidermis of *B. mori* larvae using the Total RNA Extraction Kit (Promega). Total RNA (5 μg/lane) from *B. mori* was separated by electrophoresis on a 1.0% formaldehyde agarose gel, transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany), and hybridized at 42°C with a probe in a hybridization buffer containing 5× SSC, 5× Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. Each cDNA clone of *BmGSTD2* and *BmGSTS2* was labelled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL, USA) using the Prime-It II Random Primer Labelling Kit (Stratagene, La Jolla, CA, USA), and used as probes for hybridization. After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and then exposed to autoradiography film.

Expression of recombinant protein

A baculovirus expression vector system (Je et al., 2001), using *Autographa californica* nucleopolyhedrovirus (AcNPV) and the insect cell line Sf9, was employed for the production of recombinant BmGSTD2 or BmGSTS2 protein. The *BmGSTD2* or *BmGSTS2* cDNA fragment containing the full-length open reading frame was excised from pBlueScript-*BmGSTD2* or pBlueScript-*BmGSTS2* by digestion with *Xho*I and *Sac*I, and inserted into the same sites of the transfer vector pBacPAK9 (Clontech, Palo Alto, CA, USA) in order to express BmGSTD2 or BmGSTS2 under the control of the AcNPV polyhedrin promoter. 500 ng of the construct (pBacPAK9-*BmGSTD2* or pBacPAK9-*BmGSTS2*) and 100 ng of the AcNPV viral DNA (bAcGOZA) (Je et al., 2001) were co-transfected into 1.0–1.5 × 10^6 Sf9 cells for 5 h using Lipofectin reagent (Gibco BRL, Gaithersburg, MD, USA). The transfected Sf9 cells were cultivated in TC100 medium (Gibco BRL) at 27°C for 5 days. The recombinant AcNPV was propagated in Sf9 cells, and the titer was expressed as plaque forming units (PFU) per milliliter according to standard methods (O'Reilly et al., 1992).

SDS-polyacrylamide gel electrophoresis (PAGE)

Insect Sf9 cells were mock-infected or infected with the wild-type AcNPV and recombinant AcNPV in a 35 mm diameter dish (1 × 10^6 cells) at a multiplicity of infection (MOI) of 5 PFU per cell. After incubation at 27°C, cells were harvested at 2 days post-infection (p.i.). For SDS-PAGE (Laemmli, 1970) of cell lysates, uninfected Sf9 cells and cells infected with virus were washed twice with PBS, mixed with protein sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.125% bromophenol blue) and boiled for 5 min. The total cellular lysates (5 μg/lane) were subjected to 10% SDS-PAGE. After electrophoresis, gels were fixed and stained with 0.1% Coomassie Brilliant Blue R-250.

Extraction of proteins from gut

The alimentary canals of three larvae of *B. mori* were dissected and placed in cold PBS containing 1 mM PMSF (phenylmethyl-sulfonylfluoride) and a few crystals of phenylthiourea. After washing, the alimentary canals were homogenized in PBS, the homogenate centrifuged at 10,000 × g for 10 min and the supernatant was stored at –70°C until use. The protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad). The protein samples from the gut were subjected to SDS-PAGE and western blot analysis.

Production of antibodies

The recombinant BmGSTD2 and BmGSTS2 proteins (~5 μg) were electro-eluted from the SDS-polyacrylamide gel electrophoresis (PAGE) gel, mixed with an equal volume of Freund's complete adjuvant (a total of 200 ml, Sigma) and injected into Balb/c mice (Lee et al., 2005). Two successive injections of antigens mixed with equal volumes of Freund's incomplete adjuvant (a total of 200 ml) were administered at one week intervals, beginning one week after the first injection. After the last injection, which was performed with only antigen, blood was collected three days later and centrifuged at 13,000 rpm for 5 min. The supernatant antibodies were stored at –70°C until use.

Western blot analysis

Western blot analysis was carried out using an enhanced chemiluminescence (ECL) Western Blotting Analysis System (Amersham Biosciences). The protein samples were mixed with

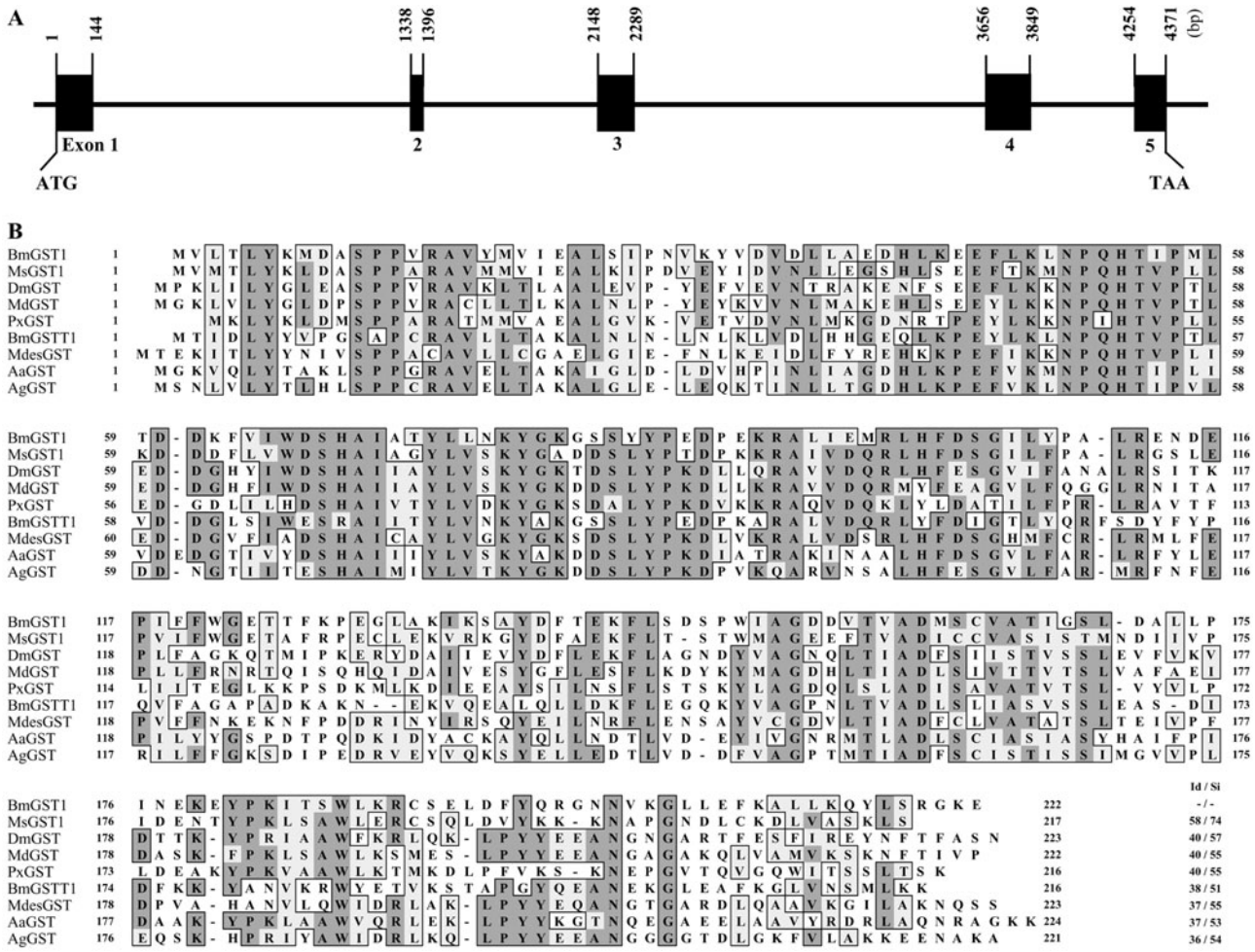

Fig. 1. Genomic organization and deduced amino acid sequence of the *BmGSTD2* gene. (A) Organization of the *BmGSTD2* gene. Numbers indicate the position in the genomic sequences. The GenBank accession number of *BmGSTD2* genomic DNA is DQ862465. (B) Alignment of the deduced amino acid sequence of *BmGSTD2* with other insect GSTs. Residues are numbered according to the aligned GST sequences and invariant residues are shaded black. The conserved G-site residues are marked with an asterisk. The closed triangle refers to the conserved serine residue at position 11. Dashes represent gaps introduced to improve alignment. The GenBank accession number of *BmGSTD2* cDNA is AY192575. The GenBank accession numbers for the aligned GST sequences are: *B. mori* GSTD2 (AY192575; this study); *Manduca sexta* (P46430); *Musca domestica* (AF147205); *Drosophila melanogaster* (NM_137485); *Plutella xylostella* (AB232916); *Mayetiola destructor* (AY518897); *Aedes aegypti* (AY819709); *Anopheles gambiae* (AF316636); *B. mori* GSTD1 (AB176691). The BmGSTD2 sequence was used as a reference for the identity/similarity (Id/Si) values.

the sample buffer, boiled for 5 min and loaded on a 10% SDS-PAGE gel (5 μg/lane). The proteins were blotted to a sheet of nitrocellulose transfer membrane (Schleicher & Schuell). After blotting, the membrane was blocked by incubation in a 1% bovine serum albumin (BSA) solution, incubated with antiserum solution (1 : 1000 v/v) at room temperature for 1 h, and washed in TBST [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% (v/v) Tween-20]. The membrane was then incubated with anti-mouse IgG horseradish peroxidase (HRP) conjugate and HRP-streptavidin complex. After repeated washing, the membrane was incubated with ECL detection reagents (Amersham Biosciences) and exposed to autoradiography film.

RESULTS AND DISCUSSION

Cloning, sequencing and analysis of *BmGSTD2* and *BmGSTS2* genes

A search of *B. mori* ESTs identified two cDNA clones that were similar to previously reported GST genes. The cDNA clones, including the full-length open reading frame (ORF), were sequenced and characterized. The *B. mori* GSTD2 (*BmGSTD2*) cDNA is 822 bp long and contains an ORF of 666 nucleotides capable of encoding a 222 amino acid polypeptide. The ORF has both a start (ATG) and stop codon (TAA), indicating that the sequence contains the complete coding region. A putative polyadenylation signal, AATAAA, was located at nucleotide positions 765–770. The *B. mori* GSTS2 (*BmGSTS2*) cDNA is 724 bp long and contains an ORF of 618 nucleotides capable of encoding a 206 amino acid polypeptide. The ORF has both a start (ATG) and stop codon (TAA) and a putative polyadenylation signal, AATAAA, located at nucleotide positions 633–638. These *BmGSTD2* and *BmGSTS2* cDNA sequences have been deposited in GenBank under accession numbers AY192575 and AY297161, respectively.

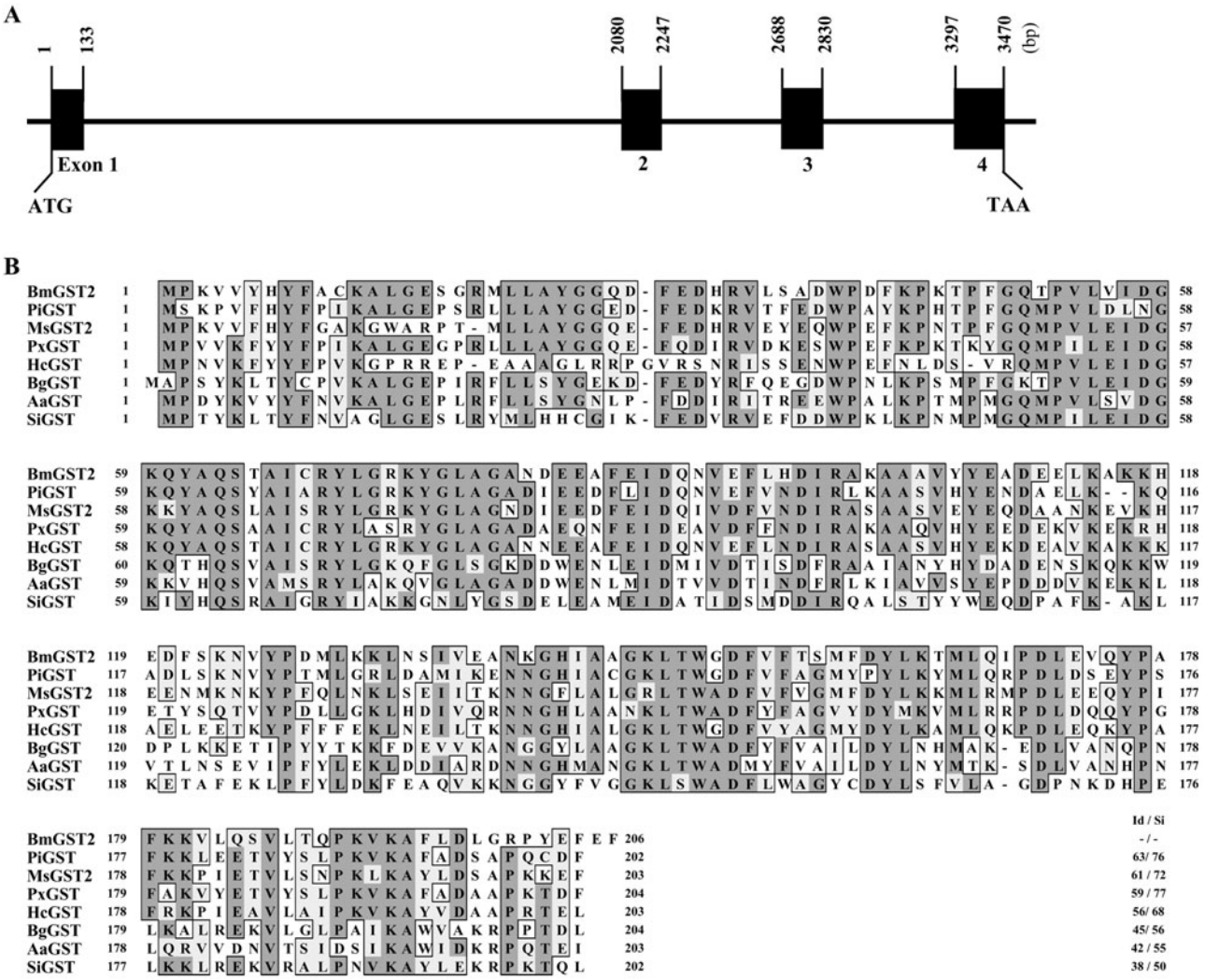

Fig. 2. Genomic organization and deduced amino acid sequence of the *BmGSTS2* gene. (A) Organization of the *BmGSTS2* gene. Numbers indicate the position in the genomic sequences. The GenBank accession number of *BmGSTS2* genomic DNA is DQ862466. (B) Alignment of the deduced amino acid sequence of BmGSTS2 with other insect GSTs. Residues are numbered according to the aligned GST sequences and invariant residues are shaded black. The conserved G-site residues are marked with an asterisk. The closed triangle refers to the conserved tyrosine residue at position 8. Dashes represent gaps introduced to improve alignment. The GenBank accession number of *BmGSTS2* cDNA is AY297161. The GenBank accession numbers for the aligned GST sequences are: *B. mori* GSTS2 (AY297161; this study); *B. mori* GSTS1 (AB206971); *Platynota idaeusalis* (AF082570); *Manduca sexta* (P46429); *Plutella xylostella* (AB180454); *Hyphantria cunea* (AB223045); *Blattella germanica* (O18598); *Aedes aegypti* (EAT36156); *Solenopsis invicta* (AY255670). The BmGSTS2 sequence was used as a reference for the identity/similarity (Id/Si) values.

To characterize the genomic structure of the *BmGSTD2* and *BmGSTS2* genes, primer sets based on the sequences of the *BmGSTD2* and *BmGSTS2* cDNAs were designed and each band was amplified from *B. mori* genomic DNA using the primers. The PCR products were cloned and sequenced. The genomic PCR product sequences were identical to the *BmGSTD2* and *BmGSTS2* cDNAs. The organization of these genes is illustrated in Figs 1A and 2A. Comparison of the genomic sequence with the sequence of the cDNA revealed that there were five exons and four introns in the *BmGSTD2* gene. The genomic sequence of the *BmGSTS2* gene indicated that there were four exons and three introns in this gene. The sequences at the exon-intron boundaries conformed to consensus eukaryotic splice sites, including an invariant GT at the intron 5' boundary and an invariant AG at its 3' boundary. The genomic DNA sizes from translation start codon to stop codon were 4371 bp for *BmGSTD2* and 3470 bp for *BmGSTS2*, respectively.

Multiple sequence alignments of the deduced protein sequences of *BmGSTD2* and *BmGSTS2* cDNA with the available insect GST sequences are shown in Figs 1B and 2B. The residues involved in the GSH-binding site of GST (G-sites) are highly conserved in the BmGSTD2 and BmGSTS2 proteins. The N-terminal domain of BmGSTD2 and BmGSTS2 possess a serine residue at position 11 and tyrosine residue at position 8, respectively. These residues are important for the catalytic mechanism of GST (Sheehan et al., 2001; Rossjohn et al., 1998). Alignment with the deduced amino acid

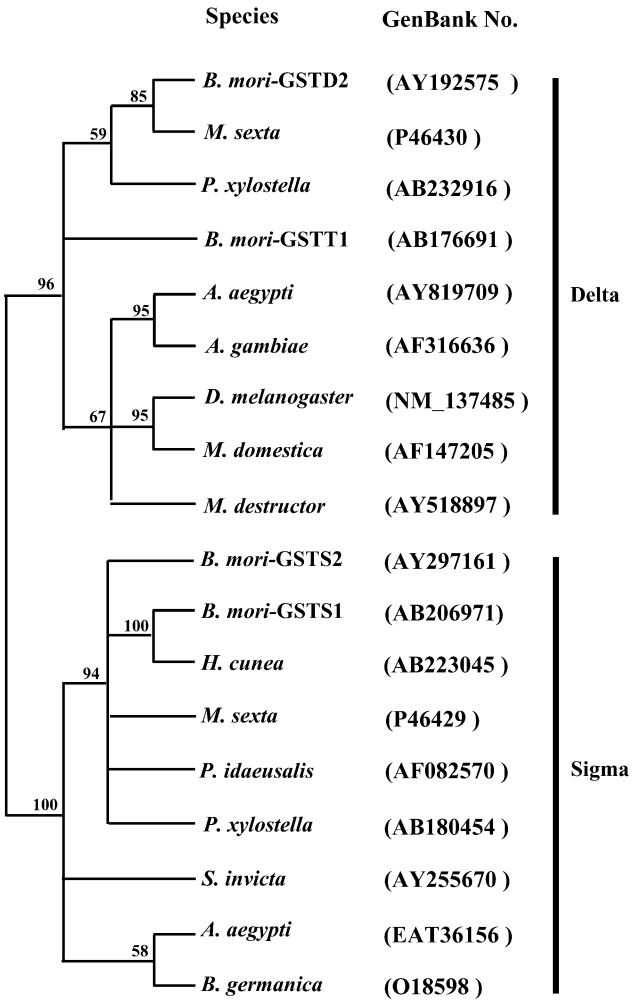

Fig. 3. Phylogenetic analysis of insect GST sequences. A maximum parsimony analysis for the amino acid sequences of BmGSTD2 and BmGSTS2 was generated with the alignment of GST sequences derived from insects. The sequence sources are described in Figs 1B and 2B. The tree was obtained by bootstrap analysis with the option of a heuristic search and the numbers on the branches represent bootstrap values for 1,000 replicates.

sequence of *BmGSTD2* cDNA indicated that the BmGSTD2 sequence was closely related to the Delta-class GST from the midgut of larvae of *Manduca sexta* (58% protein sequence identity). Alignment with the deduced amino acid sequence of *BmGSTS2* cDNA revealed that the BmGSTS2 sequence was closely related to that of *B. mori* (67% protein sequence identity), *Platynota idaeusalis* (63% protein sequence identity) and *M. sexta* (61% protein sequence identity), which all belong to the Sigma-class of GSTs. Based on the phylogenetic tree generated from the aligned amino acid sequences of insect GSTs, BmGSTD2 and BmGSTS2 were estimated to be close to Delta-class GSTs and Sigma-class GSTs, respectively (Fig. 3).

Therefore, based on sequence and structure similarity, BmGSTD2 and BmGSTS2 are proposed to belong to the Delta-class GSTs and Sigma-class GSTs, respectively. Recent studies on *B. mori* have revealed the presence of

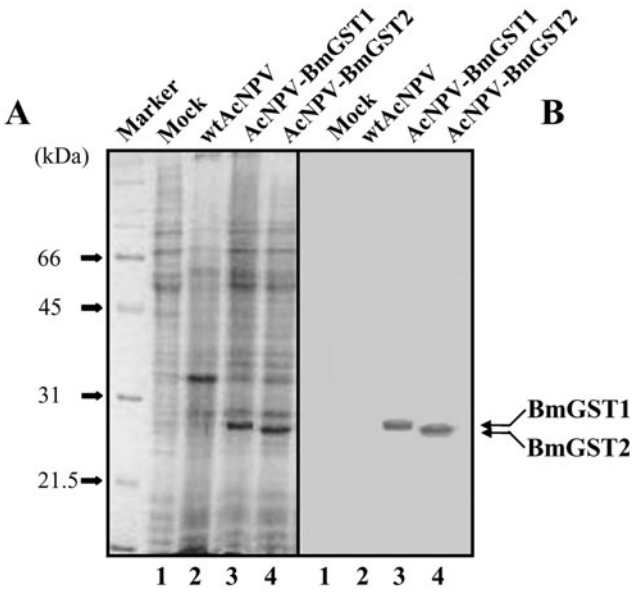

Fig. 4. Expression of the recombinant BmGSTD2 and BmGSTS2 in baculovirus-infected insect cells. Sf9 cells were mock-infected (lane 1), or infected with wild-type AcNPV (lane 2) and recombinant AcNPVs (lanes 3 and 4) at a MOI of 5 PFU per cell. Cells were collected at 2 days p.i. Total cellular lysates were subjected to 10% SDS-PAGE (A), electroblotted and incubated with recombinant BmGSTD2 or BmGSTS2 antibody (B). Molecular weight standards were used as a size marker. Recombinant BmGSTD2 and BmGSTS2 are indicated by an arrow on the right side of the panel.

GSTD1, a Delta-class GST (Yamamoto et al., 2005) and GSTS1, a Sigma-class GST (Yamamoto et al., 2006). Therefore, we named these genes *BmGSTD2* and *BmGSTS2*, respectively, in accordance with the nomenclature adopted by K. Yamamoto's group (Yamamoto et al., 2005, 2006). BmGSTS2 showed 67% protein sequence identity to the Sigma-class GSTS1 of *B. mori,* while BmGSTD2 had relatively lower identity with the Delta-class GSTD1 from the *B. mori* fat body (38% protein sequence identity). This evidence suggests that there are multiple GST isoforms in *B. mori*. This is supported by the multiple GSTs found in insect species; insect GSTs are encoded by a multigene family in flies, mosquitoes, and honeybees (Toung et al., 1993; Zhou & Syvanen, 1997; Ranson et al., 2002; Lumjuan et al., 2005; Claudianos et al., 2006).

Expression of BmGSTD2 and BmGSTS2 in baculovirus-infected insect cells

To assess the *BmGSTD2* and *BmGSTS2* cDNAs, the cDNAs were inserted into a baculovirus transfer vector. The baculovirus transfer vectors were used to generate a recombinant virus expressing BmGSTD2 or BmGSTS2. The transfer vectors, pBacPAK9-*BmGSTD2* or pBacPAK9-*BmGSTS2*, were constructed by insertion of *BmGSTD2* or *BmGSTS2* cDNA under the control of an AcNPV polyhedrin promoter of pBacPAK9 into the vector (data not shown). Recombinant AcNPVs, which we have termed AcNPV-BmGSTD2 or AcNPV-BmGSTS2, were produced in insect Sf9 cells by co-

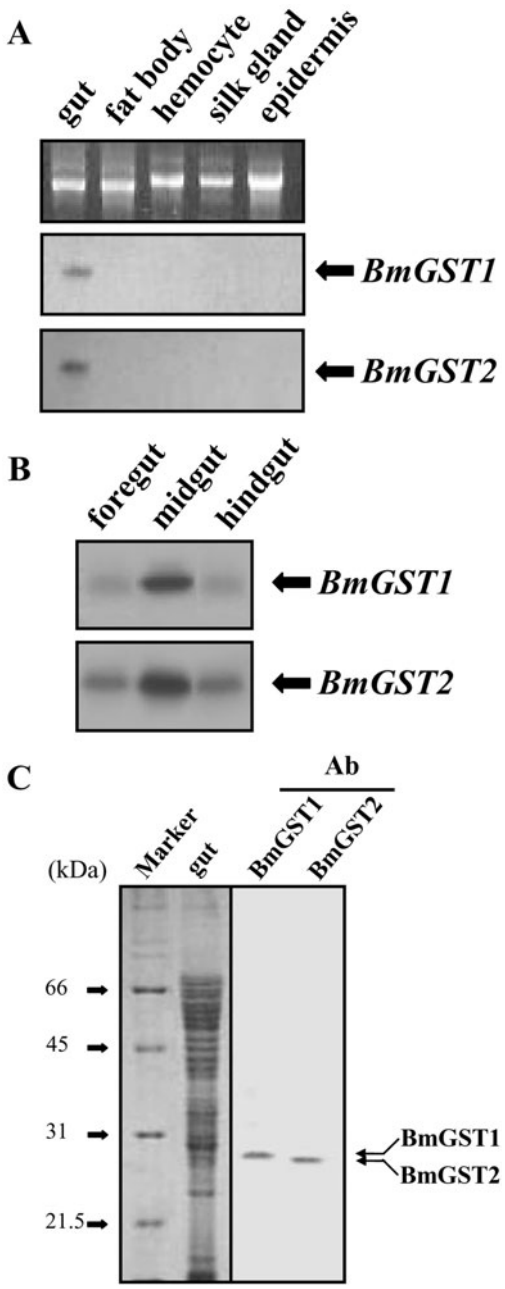

Fig. 5. Distribution of BmGSTD2 and BmGSTS2 in larvae of *B. mori*. (A) Northern blot analysis of the *BmGSTD2* and *BmGSTS2* genes. Total RNA was isolated from the gut, fat body, haemocytes, silk gland and epidermis. The RNA was separated by electrophoresis on a 1.0% formaldehyde agarose gel (upper panel), transferred onto a nylon membrane and hybridized with radiolabelled *BmGSTD2* (middle panel) or *BmGSTS2* (lower panel) cDNA. (B) Northern blot analysis of *BmGSTD2* and *BmGSTD2* genes in three gut regions. Total RNA was isolated from the foregut, midgut and hindgut, and analyzed by northern blot hybridization. Transcripts of *BmGSTD2* (upper panel) and *BmGSTS2* (lower panel) are indicated by an arrow on the right side of the panel. (C) Western blot analysis of BmGSTD2 and BmGSTS2 expressed in the guts of larvae of *B. mori*. The protein sample was collected from the guts of fifth instar *B. mori* larvae. The protein samples were subjected to 10% SDS-PAGE (left panel), electroblotted and incubated with recombinant BmGSTD2 or BmGSTS2 antibody (right panel).

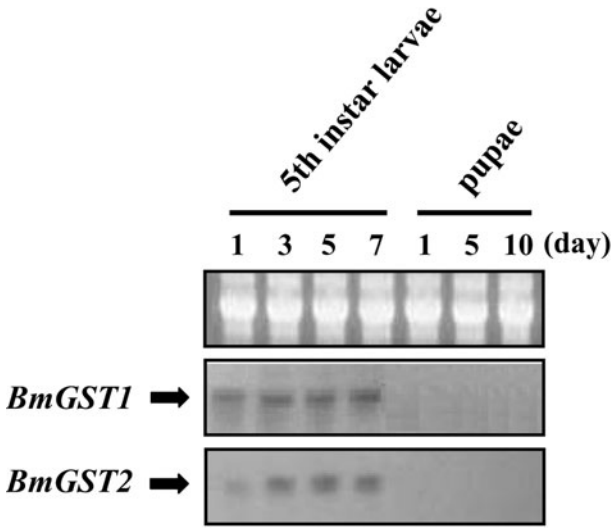

Fig. 6. Northern blot analysis of *BmGSTD2* and *BmGSTS2* in the larval and pupal stages of *B. mori*. *B. mori* guts were collected from fifth instar larvae and pupae (as indicated). Total RNA isolated from the guts was separated by electrophoresis on a 1.0% formaldehyde agarose gel (upper panel), transferred onto a nylon membrane and hybridized with radiolabelled *BmGSTD2* (middle panel) or *BmGSTS2* (lower panel) cDNA.

transfection of wild-type AcNPV DNA and the transfer vector. The recombinant BmGSTD2 or BmGSTS2 protein synthesis in Sf9 cells infected with the recombinant virus AcNPV-BmGSTD2 or AcNPV-BmGSTS2 was analyzed by SDS-PAGE (Fig. 4A). The recombinant BmGSTD2 and BmGSTS2 proteins were present as 25 kDa and 23 kDa polypeptides in the cells infected with the recombinant virus, respectively, but not in cells infected with the wild-type AcNPV or mock-infected cells. In order to further characterize the expression of BmGSTD2 and BmGSTS2, the recombinant BmGSTD2 and BmGSTS2 proteins were electro-eluted from the SDS-PAGE gel and the antibodies produced in mice. The expression of the recombinant BmGSTD2 and BmGSTS2 in baculovirus-infected insect cells was confirmed by western blotting using recombinant BmGSTD2 or BmGSTS2 antibodies (Fig. 4B). Polyclonal antibodies raised against BmGSTD2 do not cross-react with BmGSTS2, and reciprocally, antibodies raised against BmGSTS2 do not cross-react with BmGSTD2. The molecular masses of recombinant BmGSTD2 (25 kDa) and BmGSTS2 (23 kDa) were similar to most GSTs, with subunit sizes ranging from 17 to 28 kDa (Enayati et al., 2005; Lumjuan et al., 2005; Yamamoto et al., 2005, 2006, 2007).

Tissue distribution of BmGSTD2 and BmGSTS2

BmGSTD2 and BmGSTS2 distribution among the larval tissues of *B. mori* was investigated at both the transcriptional and translational levels. First, the expression of *BmGSTD2* and *BmGSTS2* was determined by northern blot analysis of the gut, fat body, haemocytes, silk gland and epidermis. Northern blot analysis demonstrated that a hybridization signal was present as a single band in the gut, indicating the gut as a specific site for BmGSTD2

and BmGSTS2 synthesis (Fig. 5A). The expression of *BmGSTD2* and *BmGSTS2* was further investigated by northern blot analysis of three different gut regions (Fig. 5B). Both *BmGSTD2* and *BmGSTS2* were expressed in all three gut regions, although the expression in the midgut was especially high when compared to the other gut regions. Next, the distributions of BmGSTD2 and BmGSTS2 were analyzed from gut protein samples using antibodies against recombinant BmGSTD2 or BmGSTS2 (Fig. 5C). A band of 25 kDa or 23 kDa corresponding to BmGSTD2 or BmGSTS2, respectively, was detected from gut protein samples in the western blot analysis. Consistent with the northern blot analysis, the results of western blot analysis revealed that both BmGSTD2 and BmGSTS2 were expressed in the guts of larvae. In addition, *BmGSTD2* and *BmGSTS2* were continuously expressed in the guts of fifth instar larvae but not detected at the pupal stage (Fig. 6).

Insect GSTs are widely distributed in all insect tissues and regulated in a tissue- or developmental-specific manner (Clark, 1989; Feng et al., 1999; Ding et al., 2003; Enayati et al., 2005; Yamamoto et al., 2005, 2006). High levels of GST activity in insects are reported in the fat body and midgut, which are important sites for the detoxification of xenobiotics. Therefore, information regarding the tissue distribution of specific GST isoforms is required in order to study the implications of GST activity in the detoxification of harmful physiological and xenobiotic electrophilic compounds. In this study, BmGSTD2 and BmGSTS2 showed high protein sequence identities to Delta-class and Sigma-class GSTs from the midgut of larvae of *M. sexta* (Snyder et al., 1995). In addition, BmGSTD2 had greater homology to Delta-class GST from the midgut of larvae of *M. sexta* (Snyder et al., 1995) than Delta-class GST from the fat body of *B. mori* (Yamamoto et al., 2005). Taken together, our results indicate that BmGSTD2 and BmGSTS2 are expressed exclusively in the gut of larvae and suggest that the expression of *BmGSTD2* and *BmGSTS2* is regulated in a tissue-specific manner.

In this study, we have reported the cloning, expression and distribution of two novel gut-specific GSTs in *B. mori*. Further investigations are required to elucidate the action and enzymatic character of BmGSTD2 and BmGSTS2.

ACKNOWLEDGEMENTS. This work was supported by a grant from the National Basic Research program of China (No. 2005CB121000) and the Rural Development Administration (Biogreen 21 program), Republic of Korea.

REFERENCES

ARRUDA L.K., VAILES L.D., PLATTS-MILLS T.A.E., HAYDEN M.L. & CHAPMAN M.D. 1997: Induction of IgE antibody responses by glutathione S-transferase from the German cockroach (Blattella germanica). *J. Biol. Chem.* **272**: 20907–20912.

CHELVANAYAGAM G., PARKER M.W. & BOARD P.G. 2001: Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. *Chem. Biol. Interact.* **133**: 256–260.

CLARK A.G. 1989: The comparative enzymology of the glutathione S-transferases from non-vertebrate organisms. *Comp. Biochem. Physiol. (B)* **92**: 419–446.

CLAUDIANOS C., RANSON H., JOHNSON R.M., BISWAS S., SCHULER M.A., BERENBAUM M.R., FEYEREISEN R. & OAKESHOTT J.G. 2006: A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Mol. Biol.* **15**: 615–636.

DING Y., ORTELLI F., ROSSITER L.C., HEMINGWAY J. & RANSON H. 2003: The Anopheles gambiae glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genomics* **4**: 35.

ENAYATI A.A., RANSON H. & HEMINGWAY J. 2005: Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol.* **14**: 3–8.

FENG Q.L., DAVAEY K.G., PANG A.S.D., PRIMAVERA M., LADD T.R., ZHENG S.C., SOHI S.S., RETNAKARAN A. & PALLI S.R. 1999: Glutathione S-transferase from the spruce budworm, Chorstoneura fumiferana: identification, characterization, localization, cDNA cloning and expression. *Insect Biochem. Mol. Biol.* **29**: 779–793.

FOURNIER D., BRIDE J.M., POIRE M., BERGE J.B. & PLAPP F.W. 1992: Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. *J. Biol. Chem.* **267**: 1840–1845.

HUANG H.S., HU N.T., YAO Y.E., WU C.Y., CIANG S.W. & SUN C.N. 1998: Molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the diamondback moth, Plutella xylostella. *Insect Biochem. Mol. Biol.* **28**: 651–658.

JE Y.H., CHANG J.H., CHOI J.Y., ROH J.Y., JIN B.R., O'REILLY D.R. & KANG S.K. 2001: A defective viral genome maintained in Escherichia coli for the generation of baculovirus expression vectors. *Biotechnol. Lett.* **23**: 575–582.

KIM S.R., LEE K.S., KIM I., KANG S.W., NHO S.K., SOHN H.D. & JIN B.R. 2003: Molecular cloning of a cDNA encoding putative calreticulin from the silkworm, Bombyx mori. *Int. J. Indust. Entomol.* **6**: 93–97.

LAEMMLI U.K. 1970: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

LEE K.S., KIM S.R., PARK N.S., KIM I., KANG P.D., SOHN B.H., CHOI K.H., KANG S.W., JE Y.H., LEE S.M., SOHN H.D. & JIN B.R. 2005: Characterization of a silkworm thioredoxin peroxidase that is induced by external temperature stimulus and viral infection. *Insect Biochem. Mol. Biol.* **35**: 73–84.

LEE K.S., JE Y.H., WOO S.D., SOHN H.D. & JIN B.R. 2006: Production of a cellulase in silkworm larvae using a recombinant Bombyx mori nucleopolyhedrovirus lacking the virus-encoded chitinase and cathepsin genes. *Biotechnol. Lett.* **28**: 645–650.

LEE K.S., KIM B.Y., JE Y.H., WOO S.D., SOHN H.D. & JIN B.R. 2007: A technique for producing recombinant baculovirus directly in silkworm larvae. *Biotechnol. Lett.* **29**: 175–180.

LUMJUAN N., MCCARROLL L., PRAPANTHADARA L., HEMINGWAY J. & RANSON H. 2005: Evaluated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, Aedes aegypti. *Insect Biochem. Mol. Biol.* **35**: 861–871.

MANNERVIK G., BOARD P.C., HAYES J.D., LISTOWSKY I. & PEARSON W.R. 2005: Nomenclature for mammalian soluble glutathione transferases. *Methods Enzymol.* **401**: 1–8.

O'REILLY D.R., MILLER L.K. & LUCKOW V.A. 1992: *Baculovirus Expression Vectors: A Laboratory Manual*. W.H. Freeman, New York, 347 pp.

ORTELLI F., ROSSITER L.C., VONTAS J., RANSON H. & HEMINGWAY J. 2003: Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector Anopheles gambiae. *Biochem. J.* **373**: 957–963.

PRAPANTHADARA L., HEMINGWAY J. & KETTERMAN A.J. 1993: Partial purification and characterization of glutathione S-transferase involved in DDT resistance from the mosquito Anopheles gambiae. *Pest Biochem. Physiol.* **47**: 119–133.

RANSON H., PRAPANTHADARA L. & HEMINGWAY J. 1997: Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of Anopheles gambiae. *Biochem. J.* **324**: 97–102.

RANSON H., ROSSITER L., ORTELLI F., JENSEN B., WANG X., ROTH C.W., COLLINS F.H. & HEMINGWAY J. 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.

RANSON H., CLAUDIANOS C., ORTELLI F., ABGRALL C., HEMINGWAY J., SHARKHOVA M.V., UNGER M.F., COLLINS F.H. & FEYEREISEN R. 2002: Evolution of supergene families associated with insecticide resistance. *Science* **298**: 179–181.

ROSSJOHN J., MCKINSTRY W.J., OAKLEY A.J., VERGER D., FLANAGAN J., CHELVANAYAGAM G., TAN K.L., BOARD P.G. & PARKER M.W. 1998: Human theta class glutathione transferase: the crystal structure reveals a sulfate-binding pocket within a buried active site. *Structure* **6**: 309–322.

SHEEHAN D., MEADE G., FOLEY V.M. & DOWD C.A. 2001: Structure, function and evolution of glutathione transferases: implications for classfication of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* **360**: 1–16.

SNYDER M.J., WALDING J.K. & FEYEREISEN R. 1995: Glutathione S-transferases from larval Manduca sexta midgut: sequence of two cDNAs and enzyme induction. *Insect Biochem. Mol. Biol.* **25**: 455–465.

SWOFFORD D.L. 2000: *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4, Sinauer Sunderland, MA.

TOUNG Y.P.S., HSIEH T. & TU C.P.D. 1993: The glutathione S-transferase D genes: a divergently organized, intronless gene family in Drosophila melanogaster. *J. Biol. Chem.* **268**: 9737–9746.

VALLES S.M., PERERA O.P. & STRONG C.A. 2003: Purification, biochemical characterization, and cDNA cloning of a glutathione S-transferase from the red imported fire ant, Solenopsis invicta. *Insect Biochem. Mol. Biol.* **33**: 981–988.

VONTAS J.G., SMALL G.J. & HEMINGWAY J. 2001: Glutathione S-transferases as antioxidant defense against confer pyrethroid resistance in Nilaparvata lugens. *Biochem. J.* **357**: 65–72.

YAMAMOTO K., ZHANG P., MIAKE F., KASHIGE N., ASO Y., BANNO Y. & FUJII H. 2005: Cloning, expression and characterization of theta-class glutathione S-transferase from the silkworm, Bombyx mori. *Comp. Biochem. Physiol. (B)* **141**: 340–346.

YAMAMOTO K., ZHANG P., 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.

ZHOU Z.H. & SYVANEN M. 1997: A complex glutathione transferase gene family in the housefly Musca domestica. *Mol. Gen. Genet.* **256**: 187–194.

Received January 15, 2008; revised and accepted February 18, 2008