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Cloning and Characterization of Two Glutathione *S*-Transferase cDNAs in the Spruce Budworm, *Choristoneura fumiferana*

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Two *Choristoneura fumiferana* glutathione *S*-transferase cDNAs were cloned from a cDNA library constructed using mRNA from the midgut cell line, CF-203. These cDNAs (*Cf*GST2, *Cf*GST3) encoded two structurally different proteins with a predicted molecular mass of 21 and 24 kDa, respectively, which was confirmed through protein expression in a bacterial system. Quantitative reverse-transcription PCR analyses revealed that the transcripts of these two genes were present in the epidermis, fat body, and midgut of the 6th instar larvae. *Cf*GST2 was expressed in the fat body when the insects were close to pupal molting, while it was constantly expressed in the other two tissues during the 6th instar stage. *Cf*GST3 gene was expressed highly and constantly in all of the tissues throughout the 6th instar stage. Immunohistochemistry analysis demonstrated that *Cf*GST2 and *Cf*GST3 proteins were present mainly in the fat body and epidermis and no protein was detected in the midgut. *Cf*GST2 and *Cf*GST3 were different from *Cf*GST reported before (Feng et al., 1999: *Insect Biochem Mol Biol* 29:779–793) in amino acid sequence, expression pattern, and responsiveness to tebufenozide. Arch. Insect Biochem. Physiol. 66:146–157, 2007.

KEYWORDS: glutathione *S*-transferase; detoxification; insecticide; resistance

INTRODUCTION

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of proteins involved in detoxification of xenobiotics, intracellular transport of hormones, endogenous metabolites and exogenous chemicals, and protection from oxidative damage. GSTs can conjugate reduced glutathione on the thiol of cysteine to various electrophiles and bind to a variety of hydrophobic substances. Many reports have demonstrated by different approaches that insect GSTs play an important role in insecticide resistance by detoxification (Motoyama and Dauterman, 1980; Clark, 1989).

GST genes in mammals have been clustered into seven groups: alpha, mu, pi, sigma, theta, delta and microsomal subunits (Jakobsson et al., 1997; Ji et al., 1992, 1995). In insects, GSTs function as homo- or heterodimeric proteins and have been classified into two major groups, GST1 and GST2, based on their immunological cross-reactivity and amino acid sequences (Grant and Matsumura, 1989; Franciosa and Berge, 1995). The GST1 members have higher identities with the theta and alpha classes of mammalian GSTs, whereas the GST2 members are closer to the mu, pi, and sigma groups.

We previously cloned a GST cDNA (*Cf*GST) from the spruce budworm, *Choristoneura furmiferana*, which is one of the most widely distributed destructive forest insect pests in North America. We char-

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acterized the *Cf*GST nucleotide structure, protein activity, localization, and developmental and stress-induced expression patterns in the spruce budworm (Feng et al., 1999, 2001). Herein we describe another two cDNAs that encoded two GST isozymes, which were structurally different from *Cf*GST. We analyzed their nucleotide structure and the spatial and temporal transcript expression patterns, as well as protein distribution, in the epidermis, fat body, and midgut of 6th instar larvae. We suggest that these different genes might have various physiological functions.

MATERIALS AND METHODS

Experimental Insects and Treatment

Third instar larvae of the spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera:Tortricidae) were maintained on artificial diet (McMorran, 1965) at 22°C, 70% relative humidity, and a photoperiod of 12 h light and 12 h dark cycle, and reared until they reached the pupal stage in the laboratory.

The nonsteroidal ecdysone agonist, tebufenozide (RH5992), was applied to 2-day old 5th instar insects using topical application (Retnakaran et al., 1997). A total of 0.5 µl of 10^{-6} M RH5992 in acetone was applied to each insect. The insects were collected at 6, 12. 24, and 48 h post treatment for RNA isolation. The controls were treated with the RH5992 solvent, acetone, alone.

Cell Culture

The *C. fumiferana* midgut cell line FPMI-CF-203 (CF-203, Sohi et al., 1993) was cultured at 28°C in 25-ml flasks with SF900 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were seeded at an initial concentration of 2×10^6 cells/ml in 25-ml flasks. The cells were harvested at day 2 after culture for RNA isolation.

Cloning and Sequencing of GST cDNAs

The putative GST cDNAs were isolated from an expressed sequence tag (EST) cDNA library, which was constructed in the Uni-ZAP XR vector by using the ZAP cDNA Gigapack II Gold Cloning Kit (Stratagene, La Jolla, CA) with mRNA isolated from CF-203 cells. The full-length cDNA sequences were then assembled by using the CAP3 program (Huang and Maddan, 1999) and confirmed by sequencing the longest target cDNA clones selected from the cDNA library. DNA sequencing was conducted using an ABI 377 capillary automatic sequencer.

Sequence Analysis

Annotation, comparison, and alignment of sequences were performed using the National Center for Biotechnology Information (NCBI) BLAST search services (Altschul et al., 1990) and Clustal Alignment Program (Higgins and Sharp, 1988) of DNASTAR (DNASTAR, Inc., Madison, WI). Alignment of the conserved domains was made against the Conserved Domain Database for protein classification in the NCBI database (Marchler-Bauer et al., 2005).

RNA Isolation and Reverse Transcription PCR

Total RNA was isolated from larval tissues or in vitro cells using the guanidinium thiocyanate method (Chomyczynski and Sacchi, 1987). For reverse transcription PCR (RT-PCR), mRNA was amplified using Superscript First Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. The primers for amplifying *Cf*GST2 were: forward primer: 5′-GGACGCGTGTCGTGTTCAGTTCATC-3′, reverse primer: 5′-GGAACAACAATTTAATCAGCTT-3′; for *Cf*GST3: forward primer: 5′-GGCACGAGGGACTGTGTGTGTGA-3′, reverse primer: 5′-CGGAATGAACGTGTGAGAGAATG-3′. The expected sizes of the PCR products were 761 and 738 bp for *Cf*GST2 and *Cf*GST3, respectively. The PCR reaction was performed as follows: denaturation at 94°C for 3 min, followed by 28 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s for each cycle. PCR products were separated in 1% agarose gels and stained with ethidium bromide.

Protein Expression, SDS-PAGE, and Western Blotting

Target cDNAs were cloned into the pPROEX HT expression vector (Life Technologies, Burlington, Canada) in fusion with a 6xHis tag. *E. coli* cells (XL1-Blue) were transformed with the recombinant plasmid DNAs (pPROEX-*Cf*GST2 and pPROEX-*Cf*GST3). Expression of the recombinant protein was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM.

Protein samples were denatured at 100°C for 3 min in an equal volume of 2× protein loading buffer (0.1 M Tris buffer, pH 6.8, 4% SDS, 0.2% β-mercaptoethanol, 40% glycerol, and 0.002% bromophenol blue). SDS-PAGE was performed in 7.5% acrylamide gels in Tris-glycine-SDS buffer (10 mM Tris, 50 mM glycine, 0.1% SDS, pH 8.0). The gel was stained with Coomassie Blue R-250. The molecular mass of the proteins was calculated using the GeneTools program from Syngene (Synoptics Ltd., Cambridge, UK).

For Western blot analysis, proteins were transferred from the acrylamide gel to a nitrocellulose membrane. The membrane was blocked with 3% BSA in 1× PBS buffer for 30 min at room temperature, and then incubated with the GST antibodies (1:500) at room temperature for 1 h. Goat anti-rabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody at a dilution of 1:2,000. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for color development.

Antibody Production and Immunohistochemistry Localization

The recombinant GST proteins were excised from SDS-PAGE gels. Polyclonal antiserum was made in rabbits through Cedarlane Inc. (Hornby, Ontario, Canada). Antiserum was collected after three boost injections, each with 200-ng proteins in Freund's adjuvant. Pre-immune serum collected from the same rabbit prior to immunization was used as a control.

Immunohistochemistry localization of *Cf*GST2 and *Cf*GST3 was performed as described in Feng et al. (2001). Five-day-old 6th instar larvae were fixed in 4% formaldehyde overnight at 4°C. The larvae were then embedded in paraffin and 5-μm-thick sections were made for immunostaining. The sections were stained first with the primary antibody, anti-*Cf*GST2 or anti-*Cf*GST3, at a dilution of 1:100, and then the secondary antibody, goat anti-rabbit IgG conjugated with SABC-FITC (StreptAvidin-Biotin Complex-fluorescein isothiocyanate) according to the manufacturer's instruction (Boster, Wuhan, China) at a dilution of 1:100. The sections were counter-stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) for 30 min and examined under a Leica fluorescence microscope (DMI 4000B). The photographs were taken as double exposures using fluorescence and DAPI filters.

RESULTS

Cloning of *C. fumiferaran* GSTs

Through an expressed sequence tags (ESTs) project with a cDNA library of CF-203 cells, we identified nine ESTs (out of 1,776 ESTs) that coded four GST isozymes. Among these ESTs, four were assembled into two unique transcripts that showed high identities to insect GSTs from different species (this report), while another five coded another two GSTs (unpublished data). All of these GSTs differed from *Cf*GST cloned previously from the same insect (Feng et al., 1999). These findings led us to clone and sequence the full-length sequences of the first two GST cDNA clones, named *Cf*GST2 and *Cf*GST3, respectively (Fig. 1). *Cf*GST2 was 804 nucleotides in length and coded for a 189–amino acid protein with a predicted molecular mass of 21 kDa and a pI of 5.43 (Fig. 1A). *Cf*GST3 was 983 nucleotides in length and coded for a 215–amino acid protein with a predicted molecular mass of 24 kDa and a pI of 6.62 (Fig. 1B). Sequence alignment with homologues of other insect GSTs indicated that both of them appeared to be full-length cDNA sequences and coded for complete open reading frames. There was no potential

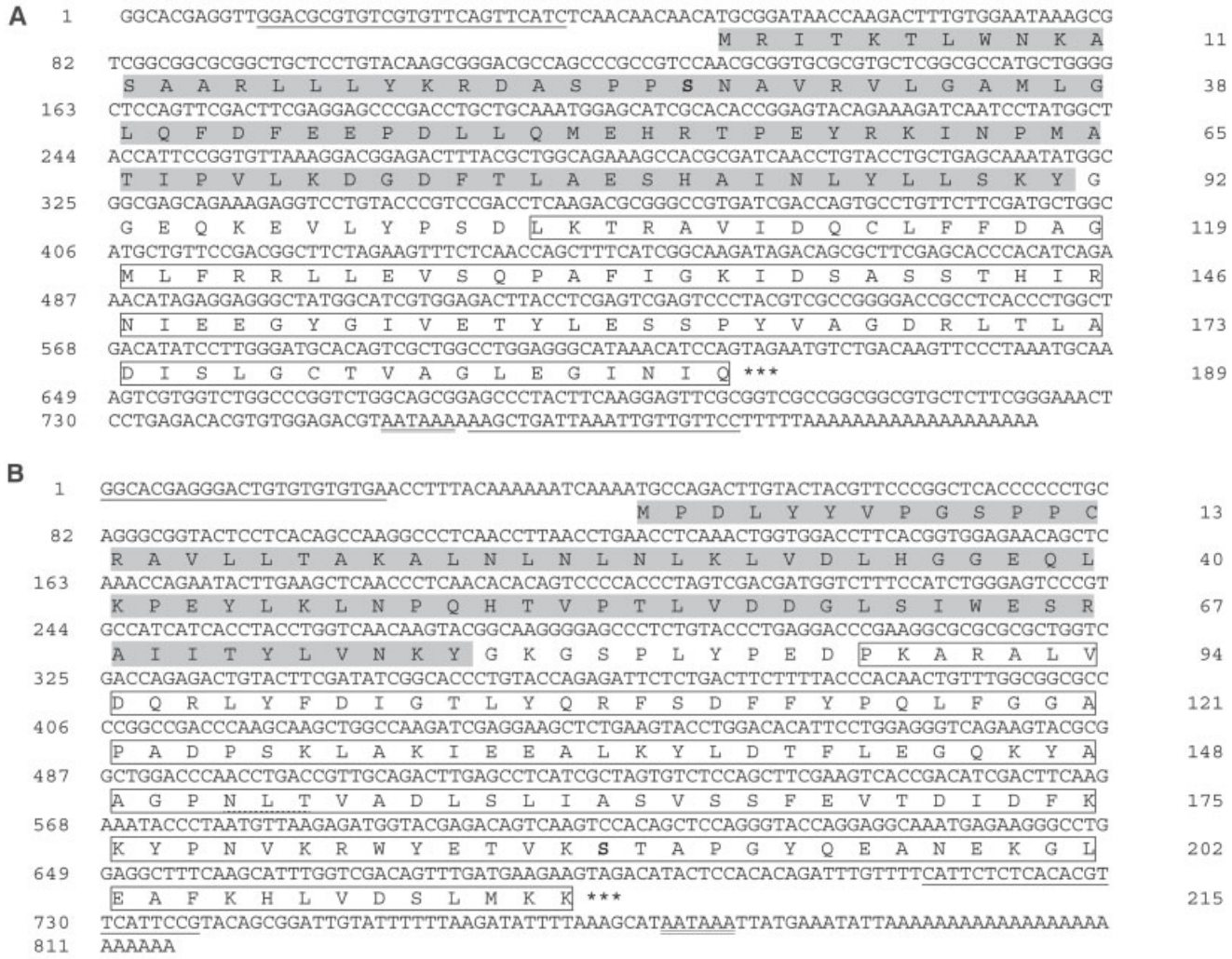

Fig. 1. Nucleotide and deduced amino acid sequences of the *Cf*GST2 (**A**) and *Cf*GST3 (**B**) cDNAs cloned from *C. fumiferana*. The numbers on the left represent the nucleotide sequence while the numbers on the right represent the amino acid sequence. The stop codon TAG is underlined with stars. The putative polyadenylation signals (AATAA) are double-underlined. The primer sequences for RT-PCR are underlined. The putative *O*-glycosylation sites are bolded (A and B) and the putative *N*-glycosylation site for *Cf*GST3 is dot-underlined (B). The putative glutathione binding region (G-site) is shaded and the electrophilic substrate binding region (H-site) is boxed. The GenBank accession numbers of these sequences are EF370472 for *Cf*GST2 (A) and EF370473 for *Cf*GST3 (B).

N-glycosylation site in the deduced amino acid sequence of *Cf*GST2, whereas one potential *N*-glycosylation site (NLT at 152) was predicted in *Cf*GST3 by using NetNGlyc 1.0 software (http://www.cbs.dtu.dk/services/NetNGlyc). There was one potential *O*-β-glycosylation site each in *Cf*GST2 (Ser27) and *Cf*GST3 (Ser189) predicted by using the YinOYang program (http://www.cbs.dtu.dk/services/YinOYang).

Comparison of *Cf*GST sequences

Sequence comparison revealed that *Cf*GST2 and *Cf*GST3 had an identity of 34.4% at the amino acid level (Fig. 2). *Cf*GST2 and *Cf*GST3 had only 14.3 and 7.9% amino acid identities, respectively, with *Cf*GST previously cloned from the same insect (Feng et al., 1999). *Cf*GST2 was 13 amino acid longer than *Cf*GST3 in the glutathione binding re-

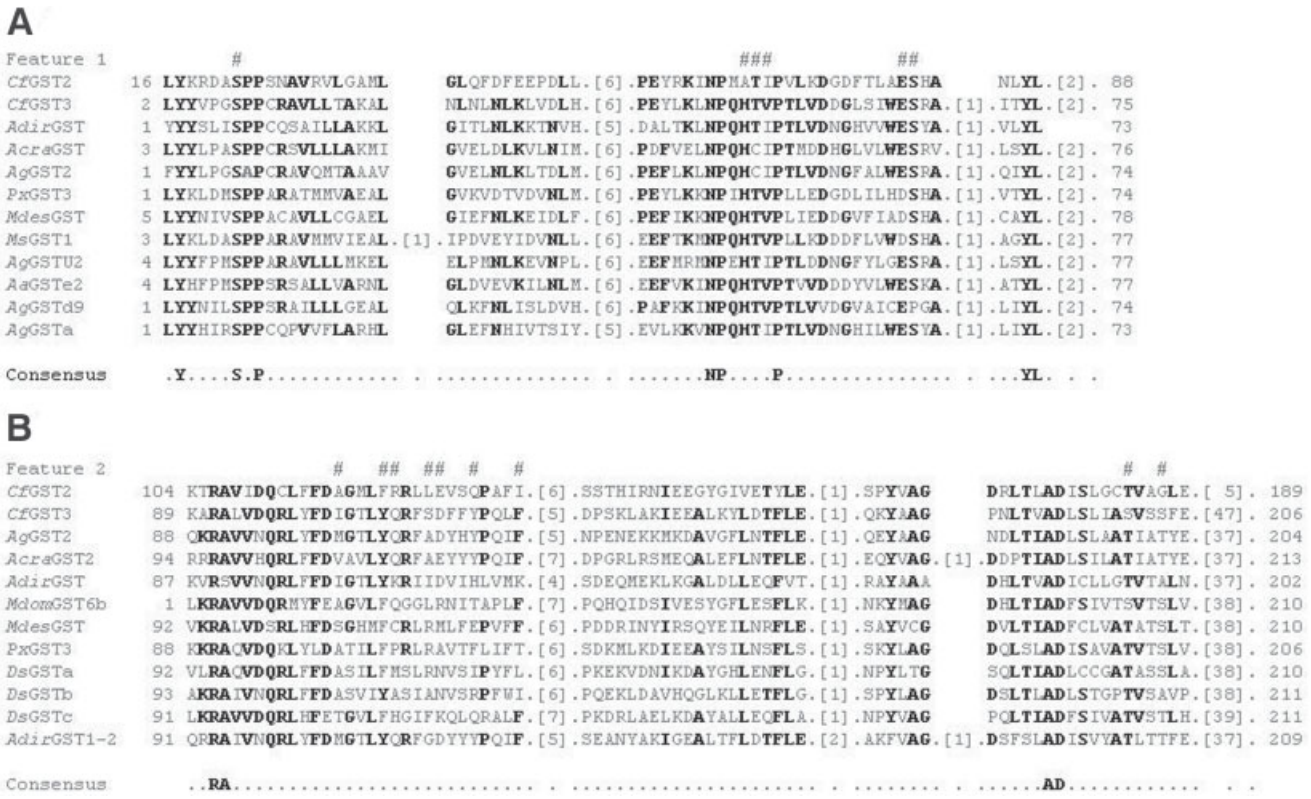

Fig. 2. Amino acid sequences alignment of the G-site region (**A**) and H-site region (**B**) of *Cf*GST2 and *Cf*GST3 with other insect GSTs. When amino acid residues in all of the sequences match, they are shown in the consensus. Otherwise, they are shown as ".". The amino acid residues that are conserved (at least 6 out of 11 sequences) among the selected GST sequences are bolded. Numbers in square brackets within each sequence are the number of amino acids that are deleted to bring the sequences into alignment. The feature amino acid residues for the G-site and H-site based on *Adir*GSTD5-5 (Udomsinprasert et al., 2005) were labelled with "#" in the top row. *Adir*GST: *A. dirus* GST (Udomsinprasert et al., 2005; 1V2A_D); *Acra*GST: *A. cracens* GST (Oakley et al., 2001; 1R5A_A); *Ag*GST2: *A. gambiae* GST2 (Chen et al., 2003; 1PN9_B); *Px*GST3: *P. xylostella* GST3 (Huang et al., 1998; AAC35245); *Mdes*GST: *Mayetiola destructor* GST (Yoshiyama et al., 2004; AAR 99711.1); *Ms*GST1: *M. sexta* GST1 (Snyder et al., 1995; P46430); *Ag*GSTU2: *A. gambiae* GSTU2 (Ding et al., 2003; AAM61890); *Aa*GSTe2: *Aedes aegypti* GSTe2 (Lumjuan et al., 2005; AAV68398); *Ag*GSTd9: *A. gambiae* GST d9 (Ding et al., 2003; AAP13483); *Ag*GSTa: *A. gambiae* GST (EAA09200); *Acra*GST2: *A. cracens* GST2 (Oakley et al., 2001; 1JLW_B); *Mdom*GST6b: *Musca domestica* GST6b (AAD54938.1); *Ds*GSTa: *D. melanogaster* GST (AAF57700); *Ds*GSTb: *D. melanogaster* GST (AAF57701); *Ds*GSTc: *D. melanogaster* GST (AAF57702); *Aa*GST: *Adir*GST1-2: *A. dirus* GST1-2 (Jirajaroenrat et al., 2001; AAG38504).

gion (G-site) (Reinemer et al., 1991) at the *N*-terminal end, but in the electrophile binding region (H-site) at the *C*-terminal end *Cf*GST3 had a 17–amino acid longer sequence than *Cf*GST2. Alignment of the conserved G-site and H-site domains of these two GSTs with those of the most identical GST sequences in the Conserved Domain Database in NCBI (Marchler-Bauer et al., 2005) revealed that *Cf*GST3 was more conserved than *Cf*GST2 in these domains. Among the top ten most identical sequences to *Cf*GST2 and *Cf*GST3, the G-site appeared to be more conserved than the H-site; for example, eight identical amino acid residues in all sequences were aligned in the G-site, whereas only two identical and adjacent (AD) amino acid residues were aligned in the H-site. There are six amino acid residues that were suggested to be the signature residues of the G-site domain in *Anopheles dirus*

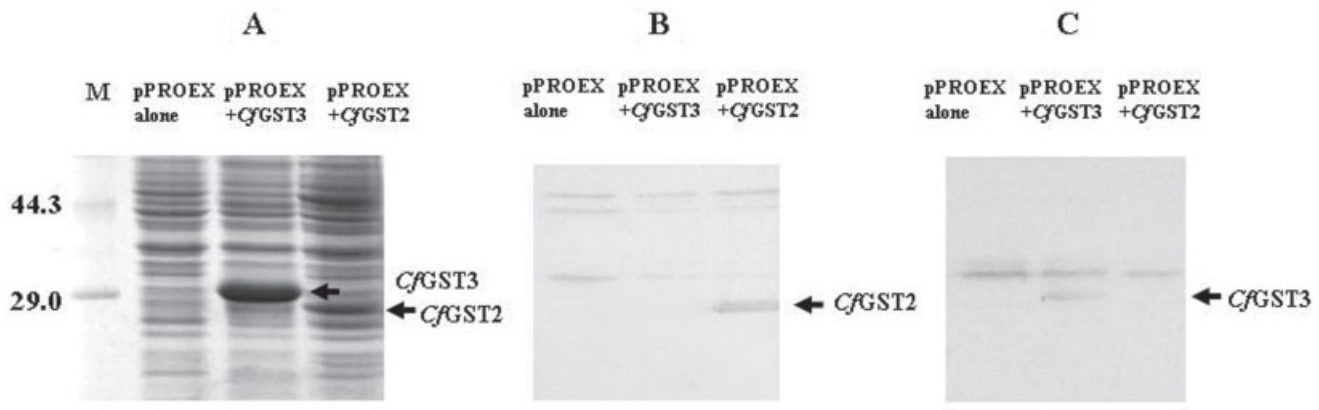

Fig. 3. SDS-PAGE (**A**) and Western blotting (**B,C**) analysis of in vitro expressed *Cf*GST2 and *Cf*GST3 proteins in the *E. coli* expression system. The expression vector was pPROEX; the recombinant expression vectors were pPROEX-*Cf*GST2 and pPROEX-*Cf*GST3. The apparent molecular masses were estimated as 27 kDa for *Cf*GST2 and 25 kDa for *Cf*GST3. *Cf*GST2 and *Cf*GST3 antibodies specifically recognize *Cf*GST2 and *Cf*GST3, respectively (B and C).

GSTD5-5 (Udomsinprasert et al., 2005), while there are nine signature residues in the H-site. *Cf*GST2 had five amino acid residues and *Cf*GST3 had nine amino acid residues matching these feature residues.

Protein Expression in a Bacterial System

The *Cf*GST2 and *Cf*GST3 cDNA open reading frames were expressed in *E. coli* into recombinant proteins fused with the 6xHis-tag (Fig. 3). The apparent molecular mass of *Cf*GST2 was 29 kDa (Fig. 3A) and the apparent molecular mass of *Cf*GST3 was 27 kDa (Fig. 3B). Because the 6xHis-tag and flanking sequences were about 2 kDa in size, the apparent sizes for *Cf*GST2 and *Cf*GST3 proteins were estimated as 27 and 25 kDa, respectively. Thus, the apparent molecular mass was 6 kDa larger than the predicted size for *Cf*GST2 (21 kDa), and 1 kDa larger than that for *Cf*GST3 (24 kDa) based on the deduced amino acid sequences of the cDNAs (Fig. 1). These results confirmed that each of the cDNAs did code for a GST protein. It is not clear why *Cf*GST2 had a higher apparent molecular mass than *Cf*GST3, which had a higher estimated molecular mass than *Cf*GST2. Two antibodies were generated against *Cf*GST2 and *Cf*GST3 and they could specifically recognize the recombinant *Cf*GST2 and *Cf*GST3, respectively (Fig. 3B and C).

Expression of *Cf*GSTs in CF-203 Cells

High levels of transcripts of these two GSTs were detected using RT-PCR in the CF-203 midgut cell line cultured in vitro without any treatment (Fig. 4). Because the primers for the RT-PCR were located at the 5′- and 3′-terminal ends of the cDNAs (Fig. 1) and the sizes of the PCR products were the same as the cloned cDNAs (Fig. 4), we believed that the isolated cDNA clones were the full-length cDNA sequences. This result confirmed the fact that

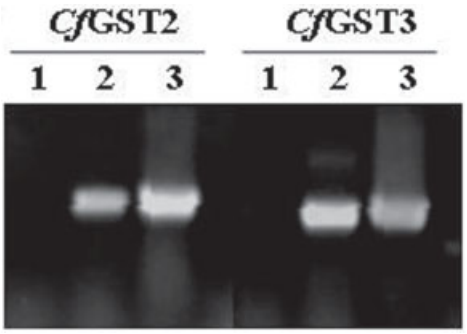

Fig. 4. Reverse-transcription PCR analysis of *Cf*GST2 and *Cf*GST3 in CF-203 cells using identical amounts of RNA. Total RNA was extracted from in vitro cultured CF-203 cells. Lane 1: Negative control, RNA samples without reverse transcriptase added; Lane 2: RNA samples with reverse transcriptase added; Lane 3: positive controls, plasmid DNA containing the target gene.

the transcripts were first identified as ESTs in CF-203 cells. The expression of these genes in in vitro cells, which were not treated with any specific compounds, suggested that although these GSTs could function as detoxifying enzymes, in this case it appears that they might play a role in maintenance of in vitro cell growth or other cellular functions.

Temporal and Spatial Expression

Expression of *Cf*GST2 and *Cf*GST3 genes were examined by using RT-PCR in 6th instar larvae feeding on artificial diet (Fig. 5). Both of *Cf*GST2 and *Cf*GST3 were constantly expressed in high levels in the epidermis of 6th instar larvae (Fig. 5A). However, in the fat body they appeared to express differently (Fig. 5B). *Cf*GST2 had only trace levels of expression during early times (before 96 h) in 6th instar larvae, but the expression levels increased when the insects approached pupal stage. *Cf*GST3 appeared to express constantly in the fat body throughout the 6th instar stage and the expression of both was highest at the prepupal stage. These two genes were constantly expressed in the midgut of 6th instar larvae (Fig. 5C). *Cf*GST3 appeared to have relatively higher expression levels than *Cf*GST2 in all of these tissues.

The developmental expression patterns detected using RT-PCR of these two GSTs indicated that (1) these GSTs were not specific to any particular tissue; instead they were expressed in all tissues; (2) *Cf*GST2 had higher expression levels in the fat body when the insects were about to molt than when the insects were in the intermolt stage; (3) *Cf*GST3 always had high and constant expression levels in all stages; (4) These two genes expressed both in larval

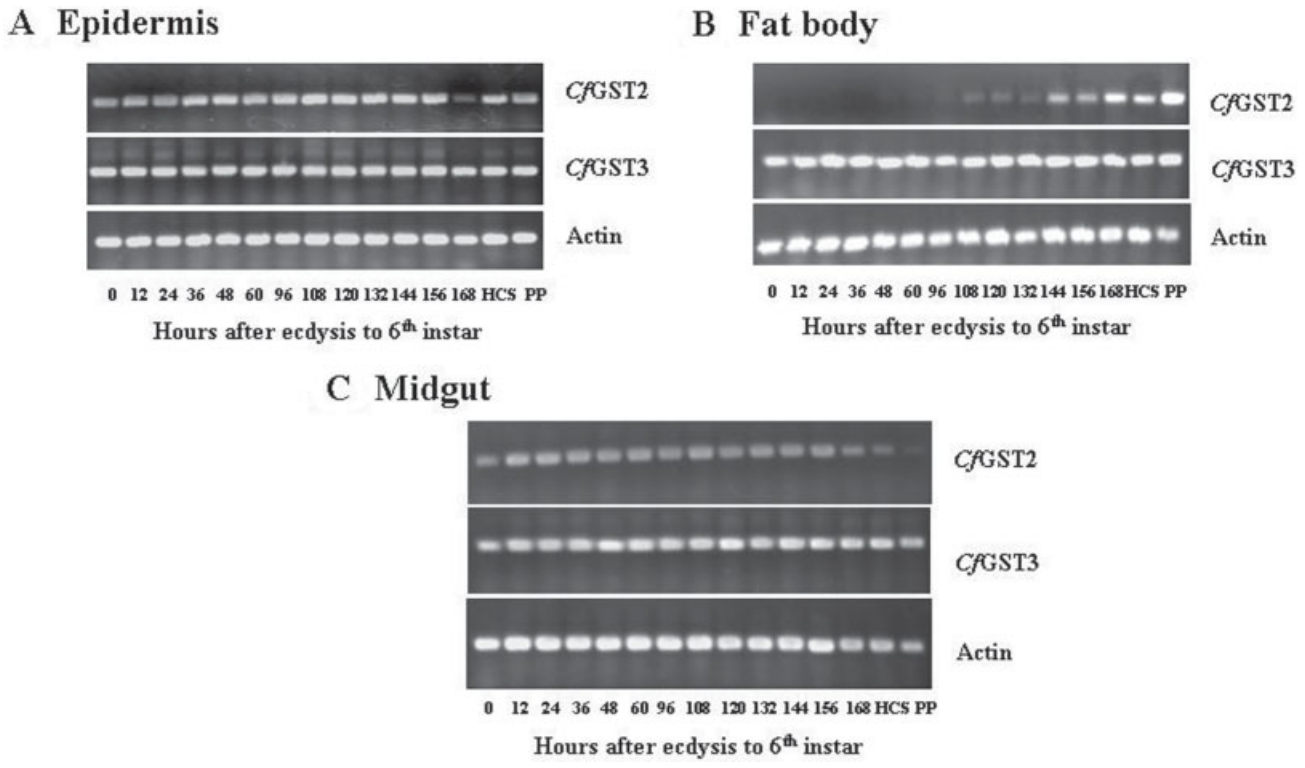

Fig. 5. RT-PCT analysis of temporal and spatial expression of *Cf*GST2 and *Cf*GST3 in the epidermis (**A**), fat body (**B**), and midgut (**C**) at different hours after ecdysis into 6th instar stage and prepupal stage. Actin expression is used as a control indicating the equal loading of RNA in the RT-PCR reactions. The sizes of PCR products of *Cf*GST2 and *Cf*GST3 are 761 and 738 bp, respectively. HCS: head capsule slippage; PP: pre-pupae.

tissues and in the cell's in vitro culture in the absence of any specific treatments.

Induced Expression by RH5992

Since *Cf*GST was induced by the ecdysone agonist tebufenozide (RH5992) in 5th instar larvae (Feng et al., 2001), we tested the effect of RH5992 on expression of *Cf*GST2 and *Cf*GST3 in 5th instar larvae (Fig. 6). The reason for using 5th instar larvae for induction expression analysis was that 6th instar larvae had constitutively high levels of *Cf*GST2 and *Cf*GST3 expression (Fig. 5), while 5th instar larvae had lower expression levels and, therefore, they were expected to be more sensitive to the induction treatment. The results indicated that at the tested concentration of 10^{-6}M, the expression of *Cf*GST2 appeared to be induced by RH5992 before 12 h post treatment in 5th instar larvae, and after 12 h post treatment the expression levels were the same as the control (Fig. 6A,B). The expression of *Cf*GST3 was not significantly induced by RH5992 before 12 h post treatment, but appeared to increase after 12 h post treatment (Fig. 6A,C).

Immunohistochemistry Localization of *Cf*GST2 and *Cf*GST3

Immunostaining analysis of *Cf*GST2 and *Cf*GST3 indicated that a large amount of *Cf*GST2 protein was found mainly in the fat body of 5-day-old 6th instar larvae (Fig. 7A). There was a low amount of

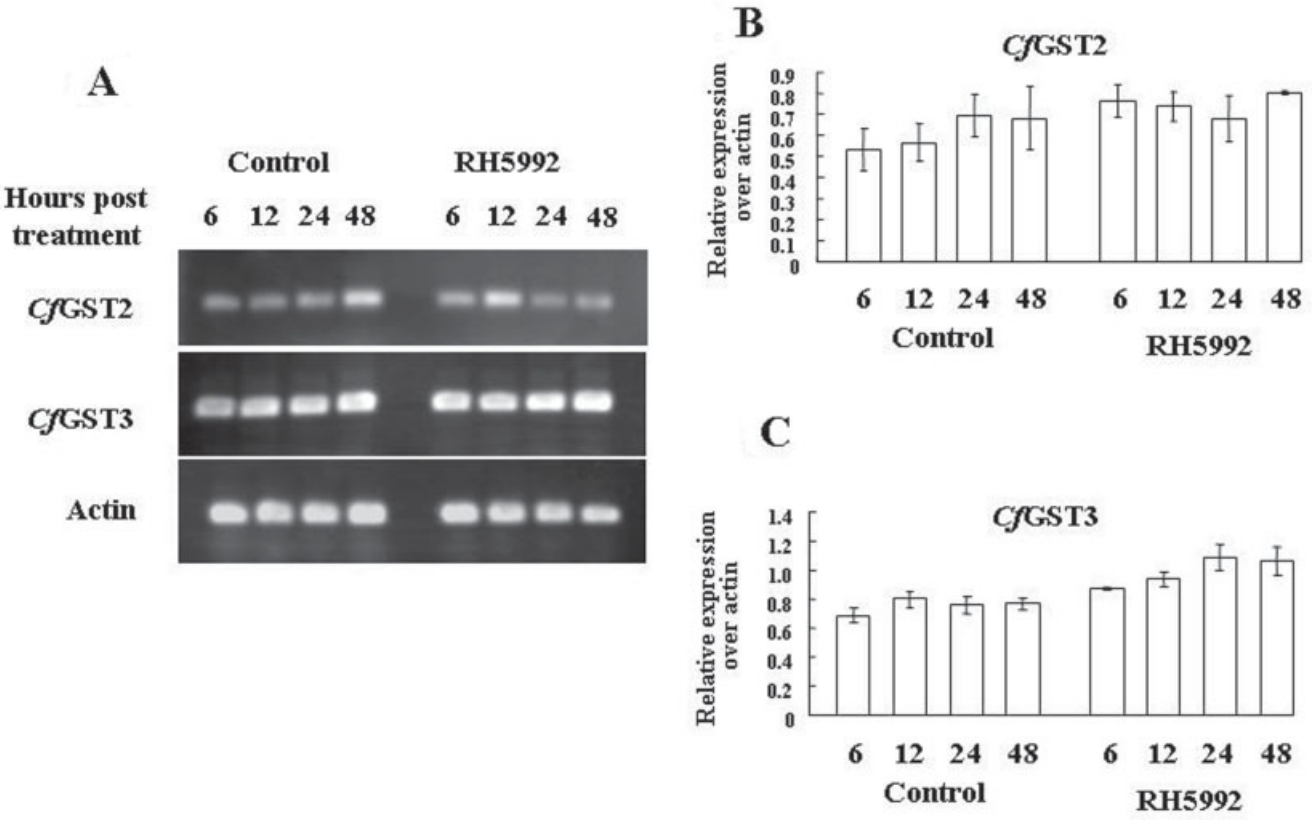

Fig. 6. **A:** Induced expression of *Cf*GST2 and *Cf*GST3 genes by the ecdysone agonist tebufenozide (RH5992) at 10^{-6}M in 5th instar larvae. Total RNA was extracted from 5th instar larvae at 6, 12, 24, and 48 h post treatment. Controls were mock-treated and samples were taken at the same times post mock treatment. Actin expression is used as a control indicating the equal loading of RNA in the RT-PCT reactions and non-responsiveness to RH5992. **B,C:** The quantitative analysis of the three repeated experiments of RT-PCR for *Cf*GST2 (B) and *Cf*GST3 (C). The PCR products were quantified using ImageQuant TL v2005 (Amersham Inc.). The relative expression of GSTs is calculated and normalized over the expression of actin.

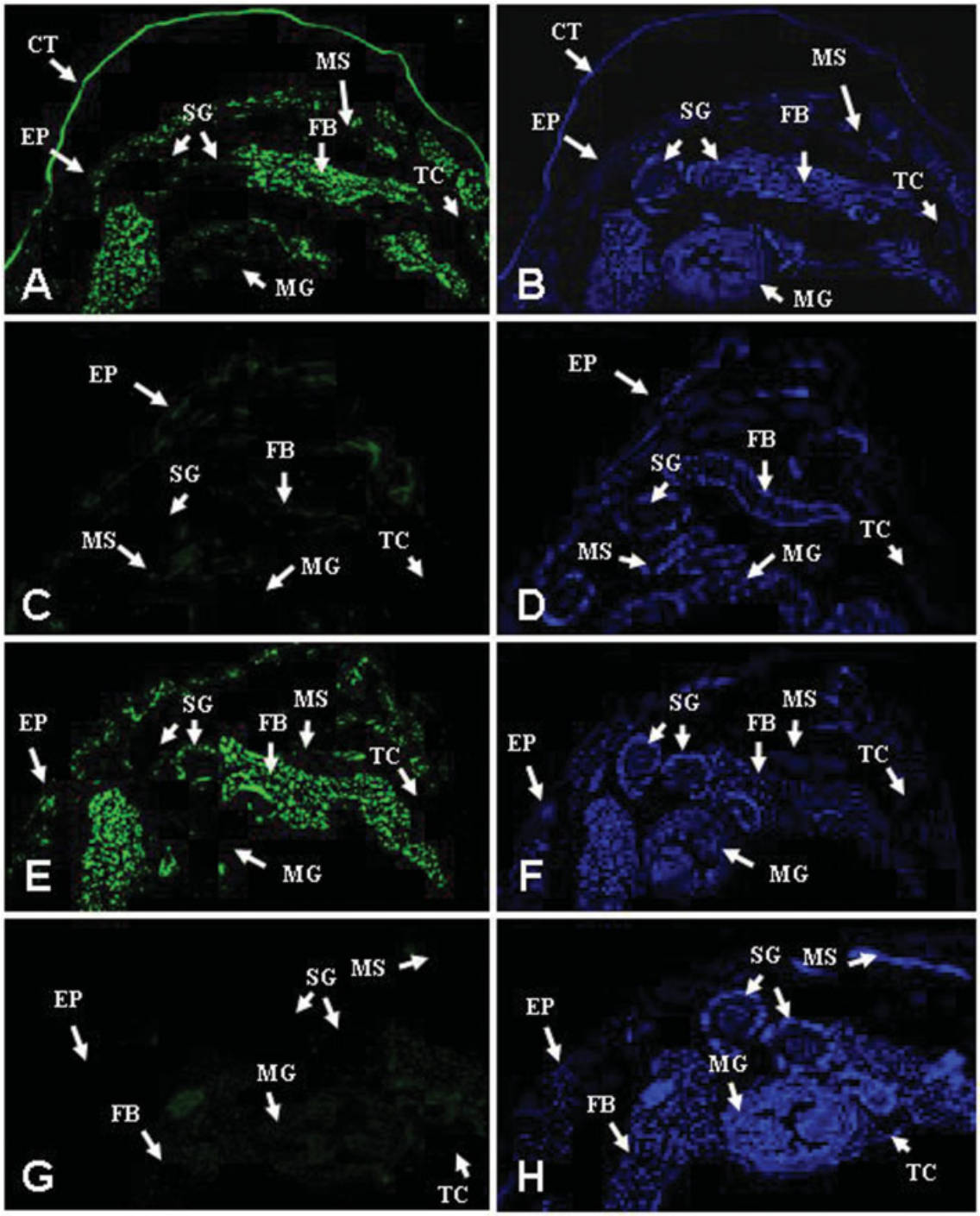

Fig. 7. Immunohistochemistry localization of *Cf*GST2 (A-D) and *Cf*GST3 (E-H) in 5-day-old 6th instar larvae. Five-micron cross-sections were immunostained with anti-*Cf*GST2 (**A,B**) or anti-*Cf*GST3 (**E,F**), or pre-immune serum from the same rabbit prior to immunization as negative controls (**C,D,G,H**), followed by goat anti-rabbit IgG conjugated with SABC-FITC and counter-stained with DAPI. The sections were observed by fluorescence microscopy and the photographs were taken as double exposure with fluorescein (A, C, E, G) and DAPI filters (B, D, F, H). FB: fat body; MG: midgut; EP: epidermis; SG: salivary gland; MS: muscle; CT: cuticle; TC: trachea.

*Cf*GST2 protein in the epidermis, muscle, and tracheal system. Similar protein distribution was found for *Cf*GST3 (Fig. 7E). While this protein was detected mainly in the fat body, it was also detected in the epidermis, muscle, and salivary glands in low levels. Only a trace amount of the GST protein was detected in the midgut. These results appeared to be inconsistent with the RT-PCR results, where mRNA was detected in all of the epidermis, fat body, and midgut tissues (Fig. 5).

DISCUSSION

We reported the cloning and characterization of a GST (*Cf*GST) isoform from the spruce budworm (Feng et al., 1999, 2001). In this study, we cloned two more GST cDNAs that were different from *Cf*GST in the following aspects. First, they differed from *Cf*GST in amino acid sequence. *Cf*GST2 and *Cf*GST3 had only 14.3 and 7.9% amino acid identities with *Cf*GST, respectively, while *Cf*GST2 and *Cf*GST3 were more similar to each other (34.4% amino acid identity). *Cf*GST has a basic pI of 9.04, while *Cf*GST2 and *Cf*GST3 have acidic pIs of 5.43 and 6.62, respectively. In another lepidopteran insect, *M. sexta*, two GSTs, GST1 and GST2, have been identified from the midgut (Snyder et al., 1995). *M. sexta* GST1 had the highest identities with members of the mammalian theta and alpha classes, whereas GST2 was most similar to members of the pi, alpha, and mu classes. *Cf*GST2 and *Cf*GST3 were more similar to *Ms*GST1 (32.8 and 38.6% identities, respectively) than to *Ms*GST2 (12.7 and 9.4% identities, respectively). Based on their similarities, *Cf*GST2, *Cf*GST3, and *Ms*GST1 belong to the insect GST1 group, while *Cf*GST and *Ms*GST2 belong to the insect GST2 group.

Secondly, *Cf*GST protein was synthesized and stored mainly in the fat body (Feng et al., 1999). Transcripts of *Cf*GST2 and *Cf*GST3 were present in all of the three tested tissues, the epidermis, fat body, and midgut of 6th instar larvae, and even in the midgut-derived cell line (CF-203). Proteins of *Cf*GST2 and *Cf*GST3 were present mainly in the fat body. In 6th instar larvae, *Cf*GST was expressed during the intermolt (Feng et al., 2001). *Cf*GST3 was constantly expressed in all of the tissues during the 6th instar stage. *Cf*GST2 was constantly expressed in the epidermis and midgut during the 6th instar stage, but in the fat body it appeared to only express highly when the larvae were close to the molt. The differences in the expression patterns between *Cf*GST and *Cf*GST2/*Cf*GST3 indicate that these two genes may play different physiological roles during the development of the insect.

Expression of *Cf*GST mRNA was significantly induced by the ecdysone agonist tebufenozide (RH5992) and allelochemicals (Feng et al., 2001), indicating that *Cf*GST may be involved in detoxification of toxic compounds. *Cf*GST2 showed an instant increased expression before 12 h post tebufenozide treatment, whereas *Cf*GST3 appeared not to be very sensitive to tebufenozide before 12 h post treatment and its increased expression was detected after 12 h. Thus, these GSTs may act in removal of the exogenous ecdysteroids applied to the insects.

Different isoforms, enzyme activity, and distribution of insect GSTs, particularly in lepidopteran species, have been demonstrated. In *M. sexta*, *Ms*GST1 and *Ms*GST2 were identified from the midgut and *Ms*GST1 was inducible by dietary chemicals (Snyder et al., 1995). Protein purification and isoelectric focusing analysis revealed that at least 6 different larval midgut GSTs were present in *M. sexta* (Snyder et al., 1995). Four isozymes of the cytosolic GSTs were identified in *Orthosia gothica* fed on a natural diet and a semisynthetic bean diet (Egaas et al., 1995). There are at least four active GSTs presenting in *Galleria mellonella* (Baker et al., 1994), Four isozymes were identified in the larval midgut of *Plutella xylostella* and one of them showed enzyme activity in an insecticide-resistant strain (Huang et al., 1998). In *Bombyx mori*, whose genome has been sequenced completely, at least seven GST genes have been found and they are localized in different chromosomes. In other lepidopterans such as *Trichoplusia ni*, *Heliothis virescens*, *Spodoptera frugiperda*, *Wiseana cervinata*, and *Heliothis zea*, at least one GST has been identified by different approaches. It is clear that as in other insects, such as the diptera *Drosophila melanogaster*, *Anopheles gambiae*, and *Aedes aegypti*, multiple genes and gene products of GST

isoforms are present in different tissues. The presence of multiple GST gene isoforms in an insect species probably reflects the multiple functions of GSTs in insect physiology.

It is important to clarify the locations of the different GST gene isoforms and their products, as well as enzymic activity, for understanding and elucidation of GST functions in the lepidopteran larvae. Most lepidopteran GSTs have been detected in the fat body and midgut (Snyder et al., 1995; Feng et al., 1999). This is consistent with our finding that high levels of gene expression and proteins of *Cf*GST2 and *Cf*GST3 (this study) and *Cf*GST (Feng et al., 2001) were detected in the fat body. In most reports on lepidopteran GSTs, GST activity was detected mainly in the midgut of *M. sexta* (Tate et al., 1982; Snyder et al., 1995). However, in the spruce budworm, we clearly demonstrated by immunohistochemistry that *Cf*GST (Feng et al., 1999), *Cf*GST2, and *Cf*GST3 (this study) are present mainly in the fat body. This study found that mRNA of *Cf*GST2 and *Cf*GST3 were detected in the midgut and epidermis, but proteins of these genes existed mainly in the fat body. There are several possibilities for this: (1) the transcripts are produced in all of these tissues but the proteins are synthesized, stored, and play their roles in the fat body, which is the major tissue for toxic substances metabolism. For example, in *Spodoptera exigua* larvae detoxification of cadmium and zinc by GST takes place in the fat body (Kafel et al., 2003); (2) in other tissues, low levels of GST proteins are enough for their functions, and therefore we could not detect their presence by using immunohistochemistry; (3) the transcripts detected in the midgut are due to contamination by the fat body because incomplete removal of all fat bodies from the midgut during the tissue dissection might be possible. All of these possibilities need to be further investigated.

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