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Identification, genomic organization and expression pattern of glutathione *S*-transferase in the silkworm, *Bombyx mori*

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ABSTRACT

Glutathione *S*-transferases (GSTs) are a multifunctional supergene family and some play an important role in insecticide resistance. We have identified 23 putative cytosolic GSTs by searching the new assembly of the *Bombyx mori* genome sequence. Phylogenetic analyses on the amino acid sequences reveal that 21 of the *B. mori* GSTs fall into six classes represented in other insects, the other two being unclassified. The majority of the silkworm GSTs belong to the Delta, Epsilon, and Omega classes. Most members of each class are tandemly arranged in the genome, except for the Epsilon GSTs. Expressed sequence tags (ESTs) corresponding to 19 of the 23 GSTs were found in available databases. Furthermore RT-PCR experiments detected expression of all the GSTs in multiple tissues on day 3 of fifth instar larvae. Surprisingly, we found little or no expression of most Delta and Epsilon GSTs in the fat body, which is thought to be the main detoxification organ. This may explain the sensitivity of the silkworm to certain insecticides. Our data provide some insights into the evolution of the *B. mori* GST family and the functions of individual GST enzymes.

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1. Introduction

Glutathione *S*-transferases (GSTs, EC2.5.1.18) are a superfamily of multifunctional enzymes found in almost all living organisms. These enzymes catalyze the nucleophilic attack of the tripeptide glutathione (GSH) on electrophilic centers of toxic compounds, including insecticides, arene oxides, quinones, and α,β-unsaturated carbonyl compounds (Motoyama and Dauterman, 1980; Clark and Shamaan, 1984; Vontas et al., 2001; Hayes et al., 2005). GSTs can serve as maleylacetoacetate isomerases and thiol transferases (Board et al., 1997, 2000). GSTs can also contain se-independent GSH peroxidase activity, which plays an important role in protecting against oxidative injury (Singh et al., 2001). In addition, GSTs have non-catalytic functions, mainly binding hydrophobic compounds such as drugs, hormones, and other metabolites (Hayes and Pulford, 1995).

GSTs may be microsomal, mitochondrial or cytosolic. Microsomal GSTs lie in a different superfamily of proteins known as the MAPEG superfamily (membrane-associated proteins in eicosanoid and glutathione metabolism) (Jakobsson et al., 1999). The mitochondrial GSTs are located in mammalian mitochondria and peroxisomes, but are not found in insects (Lander et al., 2004; Morel et al., 2004). Most insect GSTs belong to the cytosolic group. Based on amino acid sequence similarities and immunological relationships, seven classes of cytosolic GST have been recognized in mammalian species, designated Alpha, Mu, Pi, Omega, Sigma, Theta, and Zeta (Sheehan et al., 2001). Besides the ubiquitous Omega, Sigma, Theta, and Zeta classes, the insect specific Delta and Epsilon classes, as well as unclassified GSTs, have been identified in insects (Chelvanayagam et al., 2001; Ranson et al., 2001, 2002; Ding et al., 2003).

Insect GSTs play an important role in detoxifying insecticides. GST activities (toward model substrates 1-chloro-2,4-dinitro benzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB)) are significantly increased in OP (organophosphate) and pyrethroid resistant strains of the fall armyworm (Yu, 1992). *Plutella xylostella* GST3 exhibits relatively high activities toward DCNB and some OPs, suggesting that it might be related to OP resistance in the diamondback moth (Chiang and Sun, 1993; Ku et al., 1994; Huang et al.,

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; OPs, organophosphate insecticides.

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1998). The OP phoxim was widely used to control *Helicoverpa armigera* in China in the 1980s, and resistance to it has developed in Chinese populations (Wu et al., 1997). GST activity was also found to be significantly higher in phoxim-resistant *H. armigera*. These observations suggest a correlation between increased GST activity and insecticide resistance.

In this study, we have identified the silkworm cytosolic GSTs using the newly assembled 9× genome sequence (The International Silkworm Genome Sequencing Consortium, submitted for publication) and analyzed their genomic distribution and intron characteristics. We have searched available EST data for each silkworm GST to confirm active transcription and examined the expression patterns for all the GST genes in multiple tissues on day 3 fifth instar larvae by reverse transcription-polymerase chain reaction (RT-PCR). Our results provide preliminary insights into the evolution and functions of the silkworm GSTs.

2. Materials and methods

2.1. Identification of the Bombyx mori GST genes

To search for putative silkworm GST genes, GST protein sequences of *Drosophila melanogaster*, *Anopheles gambiae* and *Apis mellifera* were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) and used as queries to perform TBLASTN searches against the silkworm 9× genome database (Altschul et al., 1997). If a piece of genomic sequence showed even weak sequence similarity to any query sequence, its flanking regions (1 kb or longer) were extracted. Genes within the extracted sequences were predicted using BGF software (Wang et al., 2005) and Fgenesh+ (http://www.softberry.com/). The silkworm GSTs were classified according to conventional nomenclature (Chelvanayagam et al., 2001). Their numbering within each class mainly reflects the order of their submission to GenBank.

2.2. EST collection

To search for evidence of transcription of individual GST genes, a BLASTN search was conducted against the silkworm EST database downloaded from GenBank. The putative coding sequences were used as queries. A 95% or greater identity and minimum cut-off *E*-value (e−20) were employed to discriminate between duplicated genes.

2.3. Phylogenetic analysis

As well as the *D. melanogaster*, *A. gambiae*, and *A. mellifera* GSTs, six lepidopteran GSTs with functional data were also included to inform the possible functions of the *B. mori* clades. Putative amino acid sequences of GSTs were aligned using Clustal X (Thompson et al., 1997). Positions that had a high percentage of gaps (>70%) were manually trimmed. A phylogenetic tree was reconstructed using the neighbor-joining method (Saitou and Nei, 1987) implemented in MEGA 4.0 (Tamura et al., 2007). In addition, a maximum likelihood (ML) tree was reconstructed using PHYLIP 3.65 (Felsenstein, 2005) with the Jones–Taylor–Thornton model and 100 bootstrap replicates. A maximum parsimony (MP) tree was also built by PAUP* 4.0b10 (Swofford, 2002) with heuristic searches with tree bisection reconnection (TBR) and 100 random-taxon-addition replicates.

2.4. RNA extraction and RT-PCR

Total RNA was extracted from the fat body using Trizol reagent (Invitrogen, USA) and RNA concentration was determined using a spectrophotometer (Gene Spec V: HITACHI, Japan). DNA within RNA samples was digested with RNase-free DNase I. The first strand of cDNA was synthesized using M-MLV Reverse Transcriptase following the manufacturer's instructions (Promega, USA).

RT-PCR primers were designed on the basis of the coding sequences and ESTs of the silkworm GSTs (Supplemental Table S1). The silkworm cytoplasmic actin *A3* gene (forward primer: 5′-AACACCCCGTCCTGCTCACTG-3′; reverse primer: 5′-GGGCGAGACGT GTGATTTCCT-3′) was used as an internal control. PCR amplification was performed in a total reaction volume of 25 μl, containing normalized cDNA, 15 pmol of each primer, 2 mM MgCl2, 0.25 mM dNTP, 1× buffer and 2.5 units of Taq DNA polymerase. PCRs were performed with the following cycles: initial denaturation at 95 °C for 5 min; followed by 25 cycles of 1 min at 95 °C, 30 s annealing (temperatures listed in Supplemental Table S1), 1 min extension (72 °C), and a final extension at 72 °C for 10 min. The amplification products were analyzed on 1.5% agarose gels, purified from the gel, and directly sequenced using an ABI 3100 automated sequencer.

3. Results

3.1. Classification and phylogeny of B. mori GSTs

Using the amino acid sequences of *A. gambiae*, *D. melanogaster*, and *A. mellifera* GSTs as queries, we identified 23 putatively cytosolic GST genes by a local TBLASTN search of the silkworm genome sequence (Table 1). To reveal the relationships among the GSTs, we reconstructed phylogenetic trees using NJ, ML, and MP methods. Because the topologies of the three resulting trees are similar in overall structure, we show only the NJ tree (Fig. 1). The higher-level structure of the phylogeny is well supported in terms of bootstrap scores and distinguishes all the major classes. The silkworm GSTs cover all six classes (Delta, Epsilon, Omega, Sigma, Theta, and Zeta) found in other insects (Ding et al., 2003), with two that could not be readily assigned within one of the known classes being designated as 'unclassified'. Interestingly, both the latter are most closely related to the Delta classes (Fig. 1). Most of the BmGSTs sit within

Table 1
Summary of the silkworm GSTs

Gene name	Length of putative proteins	Number of ESTs	Chromosome	Old name	Accession number
GSTd1	218	2	6		AJ006502[b]
GSTd2	216	54	6	*GSTt*	AB176691[b]
GSTd3	220	3	6	*GST3*	DQ355374[b]
GSTd4	–	1	6		BGIBMGA006538[a]
GSTe1	222	22	7	*GST1*	AY192575[b]
GSTe2	215	0	26	*GST5*	DQ355376[b]
GSTe3	217	2	UN	*GST10*	EF506488[b]
GSTe4	223	1	7	*GST9*	EF506489[b]
GSTe5	229	1	7	*GST11*	EU216542[b]
GSTe6	223	0	21	*GST12*	EU216543[b]
GSTe7	–	6	19	*GST14*	EU216545[b]
GSTe8	–	0	10		BGIBMGA006639[a]
GSTo1	254	19	11	*GSTo1*	DQ311183[b]
GSTo2	256	22	11	*GST6*	DQ355373[b]
GSTo3	240	12	24	*GSTo*	DQ443293[b]
GSTo4	247	0	11	*GST13*	EU216544[b]
GSTs1	206	9	3	*GST2*	AY297161[b]
GSTs2	204	33	3	*GSTs*	AB206971[b]
GSTt1	229	2	8	*GST8*	EF506487[b]
GSTz1	215	12	25	*GST4*	DQ355375[b]
GSTz2	216	3	15	*GSTz*	EF565386[b]
GSTu1	233	2	6	*GST7*	EF423869[b]
GSTu2	216	12	UN		DQ311182[b]

The dash represents incomplete coding sequences. UN represents unknown chromosome locations.

[a] Accession number of the silkworm 9× genome database (http://silkworm.swu.edu.cn/silkdb/).

[b] GenBank accession number.

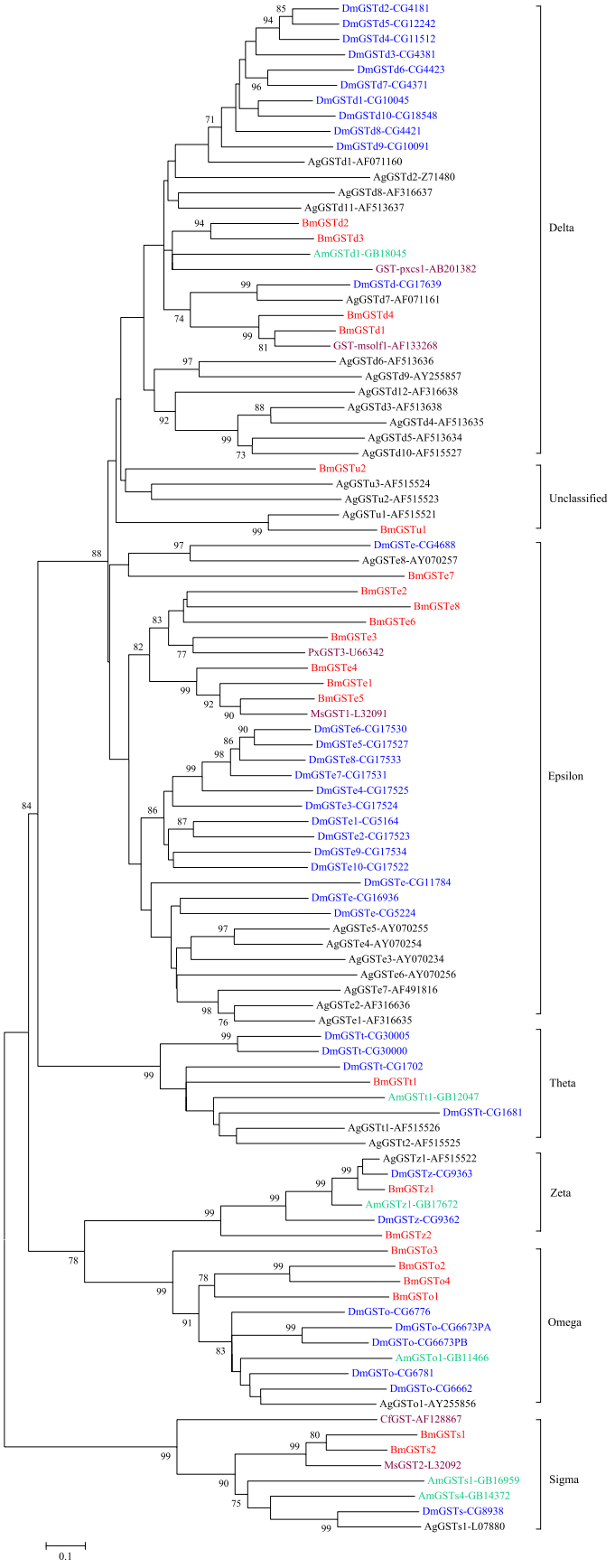

Fig. 1. Neighbor-joining consensus tree of *B. mori*, *A. gambiae*, *A. mellifera*, and *D. melanogaster* GSTs, plus some GSTs in other lepidopteran insects. Poisson correction amino acid model and pairwise deletion of gaps were selected for the tree reconstruction in the program MEGA 4.0 (Tamura et al., 2007). Bootstrap values (1000 replicates) of >70% are shown. Three incomplete ORFs of the silkworm GSTs, encoding the putative GSTd4, GSTe7, and GSTe8 proteins (200, 195, and 166 amino acids, respectively), were also included in the phylogenetic analysis. In total, an alignment of 234 amino acid sites was used to reconstruct the phylogenetic tree. *B. mori* (Bm), *A. gambiae* (Ag), *A. mellifera* (Am), *D. melanogaster* (Dm) and other lepidopteran insect GSTs were presented by red, black, green, blue, and brown, respectively. MsGST: *Manduca sexta* GST (Snyder et al., 1995); PxGST3: *Plutella xylostella* GST3 (Huang et al., 1998); CfGST: *Choristoneura fumiferana* GST (Feng et al., 2001); GST-pxcs1: preferentially expressed GST in the chemosensory organs of *Papilio xuthus* (Ono et al., 2005); GST-msolf1: olfactory-specific GST in *Manduca sexta* (Rogers et al., 1999).

Table 2
Comparison of GST gene number from the genomes of *B. mori*, *D. melanogaster*; *A. gambiae*, and *A. mellifera*

Cytosolic GSTs	*A. gambiae*	*D. melanogaster*	*A. mellifera*	*B. mori* (the % identity range within class)
Delta	12	11	1	4 (43.2–61.8)
Epsilon	8	14	0	8 (36.7–62.9)
Omega	1	5	1	4 (29.0–57.8)
Sigma	1	1	4	2 (68.1)
Theta	2	4	1	1
Zeta	1	2	1	2 (44.6)
Unclassified	3	0	0	2 (30.8)
Total	28	37	8	23

The range of amino acid identities among the *B. mori* members of each class is also presented. Only those GSTs with complete coding sequences were included in this analysis.

lepidopteran-specific subclasses and there is only one set of 1:1:1:1 orthologues between silkworm GSTs and the GSTs from other insect orders (*A. gambiae*, *D. melanogaster*, and *A. mellifera*; Fig. 1).

Compared with *A. gambiae* (12 members) and *D. melanogaster* (11 members), the silkworm Delta class is greatly reduced in number, but expanded relative to *A. mellifera* (only 1 Delta GST; Table 2) (Ding et al., 2003; Tu and Akgul, 2005; Claudianos et al., 2006). The range of amino acid identities among the silkworm GSTs in the Delta class is 43.2–61.8% (Table 2). *BmGSTd1*, *BmGSTd4*, and *GST-msolf1* form a distinct cluster, supported by a 99% bootstrap value (Fig. 1). In addition, *BmGSTd1* and the olfactory-specific *GST-msolf1* from *Manduca sexta* (Rogers et al., 1999) are closely related and share 74.4% amino acid sequence identity. They might be orthologous genes. Furthermore, *BmGSTd1* and *BmGSTd4* are arranged in tandem on chromosome 6 and have common orientations (Fig. 2.). *BmGSTd2* and *BmGSTd3* are also tandemly arranged on the chromosome 6, but in opposite orientations (Fig. 2). They form another phylogenetic group with *AmGSTd1* and *GST-pxcs1* from *Papilio xuthus*, which is preferentially expressed in the chemosensory organs (Ono et al., 2005).

Eight Epsilon GST genes were identified in the silkworm genome, whereas no Epsilon GSTs had been found in *A. mellifera* (Table 2). *BmGSTe7*, *AgGSTe8*, and *Drosophila* CG4688 might be 1:1:1 orthologues (Fig. 1). Except for the *BmGSTe7* gene, the other seven silkworm Epsilon GSTs and the other two lepidopteran Epsilon GSTs form a monophyletic clade. This suggests a lineage-specific expansion for most silkworm Epsilon GSTs, as per *A. gambiae* and *D. melanogaster*. Within the Lepidopteran *BmGSTE3* and *PxGST3* from *P. xylostella*, have 52.7% identity and could be orthologues. *PxGST3* is the first cloned GST with a well-defined role in insecticide resistance in Lepidoptera (Huang et al., 1998). *BmGSTe5* shares 65.6% identity with *M. sexta MsGST1*, which could also be orthologues (Fig. 1). *BmGSTe1*, *BmGSTe4*, and *BmGSTe5* are tandemly arranged on chromosome 7.

Four Omega GSTs were found in the silkworm genome. There are five Omega GSTs in the *D. melanogaster* genome but only one in each *A. gambiae* and *A. mellifera* (Table 2). The amino acid identities among *BmGSTo1*, *BmGSTo2*, and *BmGSTo4* are >40%, whereas the

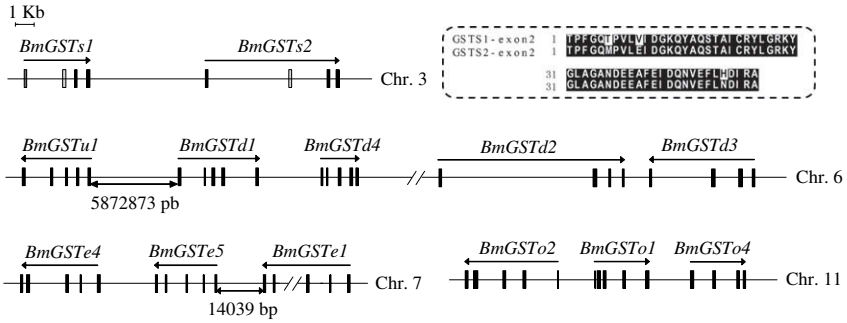

Fig. 2. The cluster organization of GST genes in the *B. mori* genome. Only those genes involved in clusters of two or more GST genes on the same scaffolds are shown. The rectangles denote the exons of the coding sequences. The two gray rectangles represent highly conserved amino acid sequences, and the protein sequence alignment is shown in the box. The arrows indicate the orientation of each gene.

identities between *BmGSTo3* and the other three silkworm Omega GSTs are about 29%. However, *BmGSTo3* shows higher level of identities (31.8–39.3%) with other insect Omega GSTs.

Zeta class GSTs are found in many different species, including plants, insects, and mammals. *B. mori*, like *D. melanogaster*, contains two Zeta GSTs whereas only one each was identified in *A. gambiae* and *A. mellifera*. The protein sequences of Zeta GSTs are highly conserved, particularly, the N-terminal SSCXWRVIAL motif (Fig. 3, Board et al., 1997). *BmGSTz1* shared 90.0%, 86.7%, and 82.5% amino acid identities with *AgGSTz1*, *CG9363*, and *AmGSTz1*, respectively, suggesting that they might be orthologous genes (Fig. 1). *BmGSTz2* only shows about 45% sequence identities with the other Zeta GSTs.

As noted earlier, the two unclassified silkworm GSTs are mostly closely related to the Delta and then Epsilon classes. The amino acid identities between *BmGSTu1* and full-length Delta and Epsilon members range from 22.0 to 34.1% (average 28.4%). Similarly, *BmGSTu2* shares amino acid identity 26.9–40.1% (average 35.3%) with the full-length Delta and Epsilon members. However, *BmGSTu1* and *BmGSTu2* showed higher levels of identity with *AgGSTu1* (65.0%) and *AgGSTu3* (40.5%), respectively. *BmGSTu1* and *AgGSTu1* might be a pair of orthologous genes. *BmGSTu2* also clustered with another two unclassified GSTs of *A. gambiae* (Fig. 1).

Both silkworm and honeybee contain one Theta class GST, fewer than were found in either *D. melanogaster* or *A. gambiae* (Table 2). Sigma class GSTs are duplicated in *B. mori* and *A. mellifera* but only present as singletons in *D. melanogaster* and *A. gambiae*. *BmGSTs1* and *BmGSTs2* are located on the same chromosome and their transcriptional orientations are identical. *BmGSTs1* shared 68.1% amino acid sequence identity with *BmGSTs2* but their second exons, each encoding 55 amino acids, only differ by three replacement changes (Fig. 2). This could reflect a relatively recent gene conversion event (Li, 1997).

3.2. Genomic distribution of GSTs in the silkworm

Most GST genes from the previously sequenced genomes show a clustered chromosomal distribution within a class. For instance, most of the Delta and Epsilon GSTs in each of *A. gambiae* and *D. melanogaster* are tandemly arranged on the same chromosome divisions (Ding et al., 2003; Sawicki et al., 2003). The silkworm Delta, Omega and Sigma classes also show strong chromosomal clustering. Overall, 21 of the 23 silkworm GSTs are distributed on 12 chromosomes, with the locations of *BmGSTe3* and *BmGSTu2* unidentified (Table 1). Delta GST genes are tandemly arranged on chromosome 6, with *BmGSTu1* also just about 5.87 Mb away (Fig. 2). The majority of Omega GSTs are located in a cluster on chromosome 11 and, two Sigma GSTs are tandemly arranged in a separate cluster on the same chromosome. However, only three Epsilon GSTs are on the chromosome in the silkworm, *BmGSTe1*, *BmGSTe5*, and *BmGSTe4*, which are tandemly located on chromosome 7 (Fig. 2).

3.3. Intron positions and sizes of GSTs

Only one intronless GST gene (*BmGSTe2*) was found in the silkworm (Fig. 4). There are also only two *A. gambiae* GSTs without introns (Ding et al., 2003). However, there are 20 intronless GSTs in *D. melanogaster* (10 Epsilon and 10 Delta GSTs; Sawicki et al., 2003). In total, at least 77 introns could be identified for the 23 silkworm GST genes (Fig. 4). Forty-three of these are phase 0 introns; phase 1 introns are present only in GSTs from non-insect specific classes. The majority of phase 2 introns were found in the Delta and Epsilon insect specific classes.

Like the *A. gambiae* GSTs, almost half of the silkworm GSTs shares a conserved intron position at approximately the 50th codon from the 5′ end of the gene (Ding et al., 2003). These GSTs include 11 from four classes: Delta, Epsilon, Sigma, and unclassified (Fig. 4). In general terms the intron positions of the silkworm members of the ubiquitous Sigma and Zeta classes are highly conserved and show very strong class-specificity. However, there is more heterogeneity within the silkworm members of the insect specific Delta, Epsilon and unclassified GSTs. Interestingly these insect specific classes also show more introns and heterogeneity in positions in silkworm than they do in the two dipteran genomes.

Intron sizes range from 72 to 13,942 bp among the silkworm GSTs, for an average of 1376 bp. About 28% of *B. mori* GST introns have sizes from 1000 to 1999 bp (Supplemental Figure S1). This is significantly bigger than intron sizes in the dipteran genomes (Ding et al., 2003). *BmGSTo3* and *BmGSTz2* contain the longest introns, with lengths of 12,421 (the first intron in the coding sequence) and 13,942 bp (the second one), respectively. Interestingly, both of them are located near the 5′ terminus of the coding sequences, so they may play roles in regulating gene expression (Duret, 2001; Haddrill et al., 2005).

3.4. Expression patterns of B. mori GSTs

To test whether the *B. mori* GSTs were actively transcribed, we searched the silkworm dbEST database downloaded from GenBank using the putative coding sequences as queries. The results indicate that multiple GSTs are expressed at each life stage, i.e. egg, larva, pupa and moth. Almost all of the silkworm GST genes match at least one EST, the exceptions being *BmGSTe2*, *BmGSTe6*, *BmGSTe8*, and *BmGSTo4* (Table 1). Overall, the non-insect specific class genes match more ESTs than did the Delta and Epsilon GSTs, albeit, *BmGSTd2* and *BmGSTe1* match 54 and 22 ESTs, respectively. Different classes show distinct expression profiles, for instance, Delta, Epsilon, Omega, Sigma, and Theta GSTs have more ESTs in

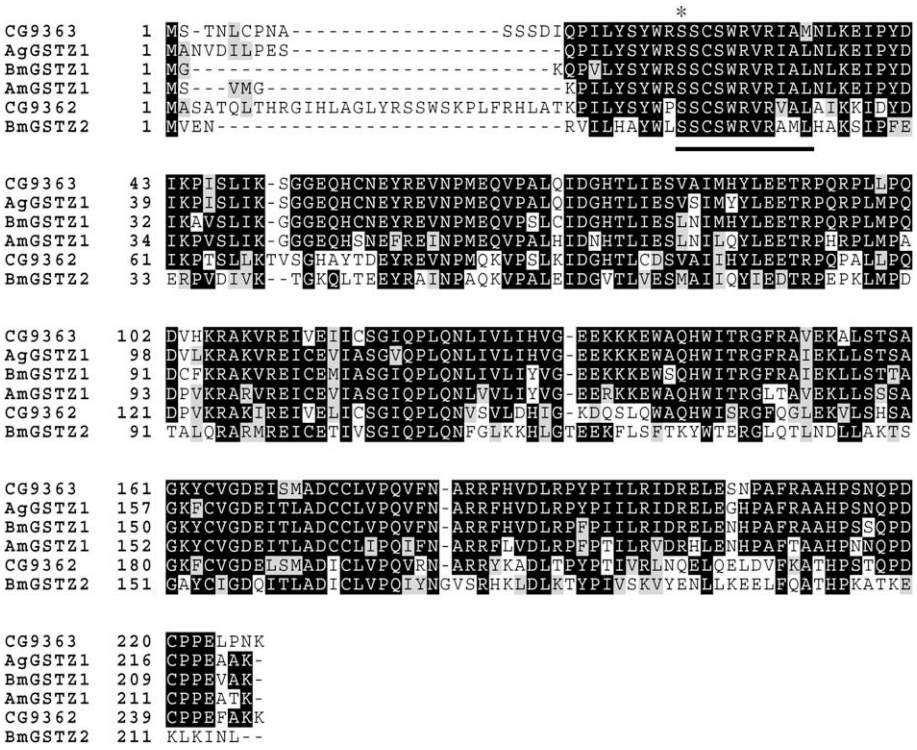

Fig. 3. Sequence alignment of insect Zeta class GSTs. Alignments were done using Clustal X with default parameters and shading was done using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Identical residues are shaded black, while similar residues are gray. The catalytic residue Ser is marked with an asterisk. The conserved motif is underlined.

non-diapause egg (19), fat body (10), silk gland (19), verson's gland (14), and malpighian tubule (2), respectively, while Zeta GSTs are more common in silk gland (3) and ovary (3) and the unclassified GSTs occur in pupae (2) and ovary (2) (Supplemental Table S2). In addition, the Delta and Epsilon classes exhibit higher expression levels in maxillary galea. These results suggest that different classes of GSTs might have different functions during development of *B. mori*.

In order to further analyze the expression of GSTs, eight tissues of day 3 fifth instar larvae were used to detect expression patterns by semi-quantitative RT-PCR. The results indicate that all 23 silkworm GSTs, including the three for which we only have partial coding sequences, are expressed in the tissues examined (Fig. 5). Most of the non-insect specific GSTs show high-level expression in various tissues, so these GSTs might play housekeeping roles in the silkworm. The unclassified GSTs are also expressed in most of the tissues. Conversely, a majority of GSTs from the Delta and Epsilon classes show more tissue-specific expression patterns. In particular, *BmGSTe2* and *BmGSTe6* ESTs were exclusively detected in midgut, albeit their expression levels were very low. Interestingly, no Delta GSTs show high-level expression in midgut and fat body and no expression at all was detected for most of the Epsilon GSTs in these tissues.

4. Discussion

Twenty-three putative cytosolic GSTs were identified in the silkworm genome. This is fewer than the corresponding figures for Dipteran insects, 37 in *D. melanogaster* (Tu and Akgul, 2005; Claudianos et al., 2006), 28 in *A. gambiae* (Ding et al., 2003) and 26 in *Aedes aegypti* (Lumjuan et al., 2007). However, it is far more than the eight that have been found in the hymenopteran *A. mellifera* (Claudianos et al., 2006). Two unclassified GSTs were found in the silkworm genome, both phylogenetically related to unclassified GSTs in *A. gambiae* (Fig. 1). In a previous study, *BmGSTd2* was classified into the Theta class (Yamamoto et al., 2005), but our phylogenetic analyses supported the later view of Yamamoto et al. (2006) that this gene should be renamed as a Delta GST.

Seventy-seven introns were identified in the 23 silkworm GSTs, at an average of 3.4 introns per gene. This is more than the number in dipteran species. For instance, only 42 introns are found in 32 GSTs, including alternative transcripts, in *A. gambiae* (1.3 introns per gene) (Ding et al., 2003). In *D. melanogaster*, 20 of 37 GST genes are intronless (Sawicki et al., 2003). The majority of intron sizes of silkworm GST genes range from 1000 to 1999 bp whereas most intron sizes of dipteran GST genes range from 50 to 99 bp (Ding et al., 2003; http://flybase.bio.indiana.edu/). Thus, silkworm GST genes contain more and longer introns than dipteran GSTs. This is probably due to the relatively high proportion of repetitive sequences in the silkworm genome (The International Silkworm Genome Sequencing Consortium, submitted for publication). The majority of these repetitive sequences are transposable elements or the remainders of transposable elements. Indeed, we did find the presence of some transposon sequences in the long introns (data not shown). Similar results were also observed in rice GSTs (Soranzo et al., 2004).

Insects have important sensilla located on their antennae. The primary function of these sensilla is to detect odours such as plant volatiles and sex pheromones. An olfactory-specific glutathione *S*-transferase gene, *GST-msolf1*, was cloned from the antennae of *M. sexta* and showed antennal-specific expression. It may play an important role in protecting the olfactory system from harmful xenobiotics (Rogers et al., 1999). A GST cDNA has also been cloned from *B. mori* antennae, which shows 74.4% amino acid identity with *GST-msolf1*, and might be orthologous. Although we have found that *BmGSTd1* is expressed in several tissues, it shows a higher level of expression in the head (Fig. 5), so it might play a similar function to the *GST-msolf1* gene in metabolizing harmful volatiles. *B. mori* is

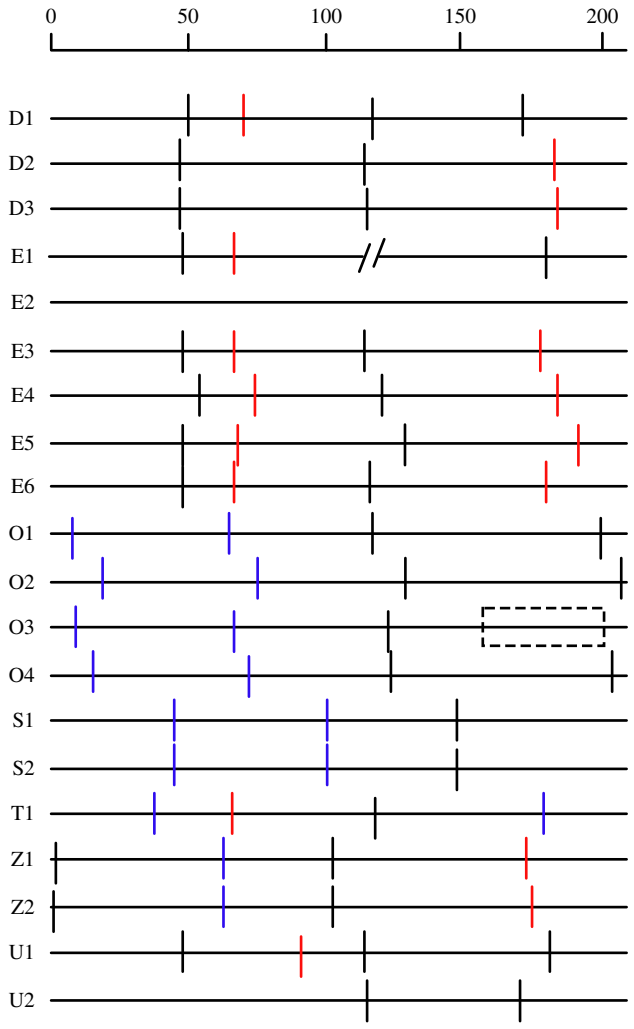

Fig. 4. Location of introns of *BmGST* genes. Phase 0, 1, and 2 introns are shown by black, blue, and red solid lines, respectively. Only those GST genes, which contained putative complete coding sequences, are shown. The boxed region of *BmGSTo3* was not found in the silkworm genome sequence, but it was obtained by PCR. The sign // within GSTE1 represents a gap in the genomic sequence. Based on the present data, we could not judge the phase of its intron.

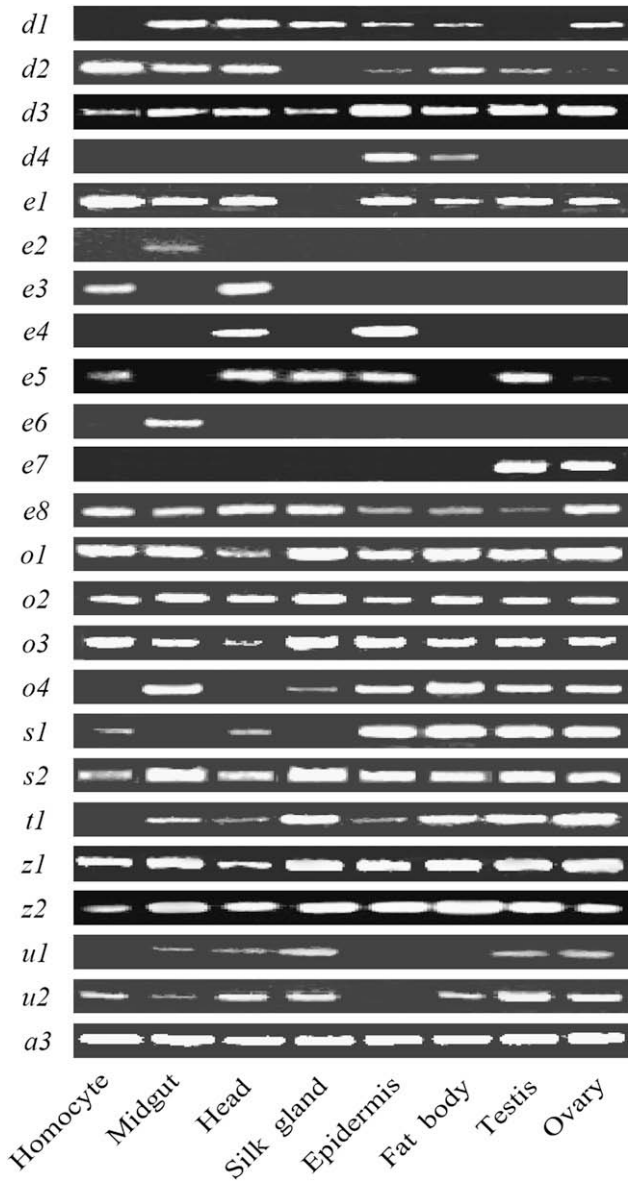

Fig. 5. Tissue expression patterns of silkworm GSTs in multiple tissues on day 3 fifth instar larvae. The silkworm cytoplasmic actin *A3* gene (*Bmactin3*; GenBank accession no. U49854) was used as an internal control and is denoted by *a3*.

an oligophagous species and feeds primarily on leaves of mulberry. Two styloconic sensilla present on each maxillary galea play an important role in gustatory function in *B. mori* (Akaoka, 2003). Maxillary sensilla and antennae show similar functions in host plant discrimination. *BmGSTd2* and *BmGSTe1* show high expression levels in the head (Fig. 5), while the EST analysis indicated that *BmGSTd2* and *BmGSTe1* have higher levels of expression in maxillary galea, seven ESTs, respectively. All these results suggest that GSTs might be important to the detoxification of volatile xenobiotics in the sensilla.

Most of the non-insect specific silkworm GSTs show high constitutive expression in various tissues (Fig. 5), suggesting that these genes may play housekeeping roles. *BmGSTs2* is widely expressed in various tissues on day 3 fifth instar larvae (Fig. 5; Yamamoto et al., 2006). *BmGSTS2* is able to conjugate GSH with 4-hydroxynonenal (4-HNE), which is the product of lipid peroxidation under oxidative stress (Singh et al., 2001). This indicates that *BmGSTs2* may play an important role in protecting against oxidative stress (Yamamoto et al., 2006).

Besides the housekeeping roles, GSTs are involved in insecticide resistance. All GSTs directly shown to be involved in insecticide detoxification belong to the insect specific Delta or Epsilon class (Li et al., 2006). In *A. mellifera*, only a single insect specific GST, *AmGSTd1*, was found. This may partially explain the extreme sensitivity of the honeybee to certain insecticides (Claudianos et al., 2006). *B. mori* is a domesticated insect and maintained in circumstances of minimal exposure to insecticide. It is also very sensitive to certain insecticides. For instance, the LC_{50} values of fifth instar NB18 silkworms to fenitrothion and ethion were only 0.306 and 0.037 mg/L, respectively (Nath et al., 1997). By comparison, another lepidopteran insect, the diamondback moth *P. xylostella*, has much greater capacity to detoxify insecticides; even third instar larvae from an insecticide sensitive strain have higher LC_{50} values for fenitrothion (196 mg/L), with the resistant strains yielding LC_{50} values that are much higher again (>1000 mg/L) (Kobayashi et al., 1992). Interestingly the silkworm has as many as 12 Delta and Epsilon GSTs, so it appears that these classes of GST are not important to OP detoxification in this species. Interestingly most of the silkworm Delta and Epsilon GSTs show little or no constitutive expression in tissues associated with detoxification such as fat body (Fig. 5).

The data presented in this study provide an overview of the genomic organization and expression profiles of the silkworm GSTs. However, little is known about the mechanisms of resistance or other biological processes of individual silkworm GSTs. Thus, further functional research of *Bm*GSTs is needed to identify key genes related to the degradation of insecticides and other xenobiotics, and mechanisms of insecticide resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.ibmb.2008.08.002.

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