Article

ANNOTATION AND EVOLUTION OF THE ANTIOXIDANT GENES IN THE SILKWORM, *Bombyx mori*

Gui-Qin Shi, Quan-You Yu, and Ze Zhang
The Institute of Agricultural and Life Sciences, Chongqing University, Chongqing, China

Ze Zhang
The Key Sericultural Laboratory of the Agricultural Ministry of China, Southwest University, Chongqing, China

Antioxidant system, which is composed of multiple gene families, plays a major role in reducing oxidative damage and xenobiotic detoxification in all living organisms. We identified 50 silkworm antioxidant genes from nine gene families based on the assembled genome sequence. A comparative analysis of the antioxidant genes of the silkworm with other order insects Anopheles gambiae, Apis mellifera, Drosophila melanogaster, and Tribolium castaneum, was performed. We found that most of the antioxidant gene families are highly conserved but Catalase (CAT) and heme-containing peroxidase (HPX) families were lineage-specifically expanded in the silkworm. The expression patterns of the silkworm antioxidant genes were investigated with the known ESTs, microarray data, and reverse transcription-polymerase chain reaction (RT-PCR). Forty two of the 50 silkworm antioxidant genes were transcribed and most of the transcribed genes showed tissue-specific expression patterns. More than a half of lineage-specifically expanded BmCATs lacked 15 or more than 15 of the 36 heme-binding residues and might lose catalase activities. However, the genes encoding these BmCATs showed almost a ubiquitous tissue expression pattern, indicating that they might have evolved new functions. In addition, the lineage-specifically expanded BmHPXs could function in maintaining cell homeostasis in the process of the synthesis of large amounts of silk proteins because they were predominantly expressed in silk

Additional Supporting Information may be found in the online version of this article.
Grant sponsor: National Science Foundation of China; Grant number: 30970409.
Correspondence to: Ze Zhang, The Institute of Agricultural and Life Sciences, Chongqing University, Chongqing 400044, China. E-mail: zezhang@cqu.edu.cn

ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY, Vol. 79, No. 2, 87–103 (2012)
Published online in Wiley Online Library (wileyonlinelibrary.com).
© 2011 Wiley Periodicals, Inc. DOI: 10.1002/arch.21014

gland of the silkworm. The lineage-specific expansion of antioxidant gene families in the silkworm provides useful information for understanding evolution and functional versatility of antioxidant genes in the silkworm even Lepidopteran insects. *© 2011 Wiley Periodicals, Inc.*

Keywords: *Bombyx mori*; antioxidant genes; evolution; phylogenetic analysis

INTRODUCTION

Reactive oxygen species (ROS) are by-products of aerobic metabolism in all organisms (Wang et al., 2001). ROS are involved in cellular signaling, including gene expression regulation, signal transduction, and physiological regulation (Kumar et al., 2003). If improperly propagated or inefficiently eliminated, ROS would lead to damage of proteins, nucleic acids, and lipids in organisms (Hermes-Lima and Zenteno-Savin, 2002). To avoid oxidative damage, organisms have evolved a complex antioxidant system. The antioxidant system of vertebrates is relatively clear (Kanzok et al., 2001). A recent comparative analysis of antioxidant genes suggested that insects also have an antioxidant system similar to vertebrate animals (Corona and Robinson, 2006). In insects, the primary and secondary antioxidant enzymes have been identified. The first defense against ROS is provided by three different kinds of primary antioxidant enzymes acting directly on ROS: superoxide dismutases (SODs), catalases (CATs), and peroxidases (PRXs). Peroxidases include heme-containing peroxidases (HPXs) and the nonheme peroxidases (non-HPXs). In addition, non-HPXs also include two main subfamilies: thioredoxin peroxidases (TPXs), glutathione peroxidases (GPXs), and some of glutathione S-transferases (GSTs). The secondary antioxidant defense indirectly acts on ROS, which is composed of thioredoxin reductases (TRXRs) and methionine sulphoxide reductases (MSRs) (Kanzok et al., 2001).

Since some insect genome sequences became available, antioxidant-related genes have been identified in the honeybee *Apis mellifera* (*Ap. mellifera*), the fruit fly *Drosophila melanogaster*, and the mosquito *Anopheles gambiae* (*An. gambiae*) (Corona and Robinson, 2006). Recently, the antioxidant-related genes in the red flour beetle *Tribolium castaneum* have been identified and some antioxidant genes, such as SODs, GPXs, TPXs, and HPXs, were found to be related to insect immunity, also known as immunity-related genes (Zou et al., 2007). Previous studies demonstrated that most of insect antioxidant gene families are highly conserved in terms of phylogenetic orthologs but lineage-specific gene duplications also occurred during evolution (Corona and Robinson, 2006; Zou et al., 2007). This may reflect different oxidative pressures in different life environments of insects since insects inhabit very diverse niches. Furthermore, studies on the functions of insect antioxidant genes have mainly focused on the response of antioxidant genes to oxidative stress produced by biotic and abiotic factors (Li et al., 2007). This is because the antioxidant genes can response to a broad range of oxidative stress produced by biotic and abiotic factors, such as H_2O_2, insecticides, allelochemicals, and so on. Relatively, SOD and CAT can be induced by allelochemicals in *Spodoptera littoralis*, a lepidopteran insect (Krishnan and Kodrik, 2006; Krishnan and Sehnal, 2006). Radyuk et al. (2003) found that the transfected *D. melanogaster* S2 cells overexpressing DPx-4783 (a 2-Cys Tpx) showed higher resistance to oxidative stress caused by exposure to H_2O_2 or paraquat than the underexpressing cell line. Therefore, it is important to accumulate data on antioxidant genes from more phylogenetically separated insect orders for understanding the molecular evolution of insect antioxidant system genes.

The silkworm, *Bombyx mori*, is a fully domesticated economic insect and one of important lepidopteran model organisms. For the silkworm antioxidant genes, previous studies mainly aimed at identification of single genes induced by biotic and abiotic factors, such as bacteria, temperature, and H_2O_2 (Lee et al., 2005; Yamamoto et al., 2005a,b,c; Kim et al., 2007; Wang et al., 2008). Moreover, the activities of SOD and CAT were compared between diapause and nondiapause eggs and between embryonic and pupal stages (Zhao and Shi, 2009, 2010). However, there is no systematic annotation for the antioxidant genes in the silkworm although the newly assembled genome sequence has been already available (The International Silkworm Genome Consortium, 2008; Duan et al., 2010). Herein, we systematically identified the silkworm antioxidant genes based on the newly assembled 9× genome sequence. We also performed a comparative analysis of the antioxidant genes of the silkworm with those of other insects to look at the evolutionary characteristics of the silkworm antioxidant system. Our results suggested that Catalase (CAT) and heme-containing peroxidase (HPX) families were lineage-specifically expanded in the silkworm while other families were highly conserved among insects. Through searching available ESTs, microarray data (Xia et al., 2007) and reverse transcription-polymerase chain reaction (RT-PCR) results, we examined the expression patterns of the silkworm antioxidant genes. Our data provide some new insights into evolutionary conservation and divergence of the silkworm antioxidant genes as well as the molecular evolution of antioxidant system in holometabolous insects.

MATERIALS AND METHODS

Identification of B. mori Antioxidant Genes

Complete protein sequences of antioxidant enzymatic genes of *D. melanogaster*, *An. gambiae*, and *Ap. mellifera* were downloaded from the GenBank (http://www.ncbi.nlm.nih.gov/), and were used as queries to search for candidate antioxidant genes using the tblastn program against the silkworm 9× genome database (The International Silkworm Genome Consortium, 2008). The antioxidant gene domains in the protein sequences encoded by the candidate genes were identified using the program SMART and the Conserved Domain Architecture Retrieval Tool (CDART) (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi). The same methods were used for identification of the *T. castaneum* antioxidant genes. If some genome sequences showed even weak sequence similarity to any query sequences, their flanking regions (1 kb or more long) were manually extracted, and then put them into Softberry database to predict new genes using FGENESH program. The silkworm and *T. castaneum* antioxidant genes were classified according to *Drosophila* and honeybee orthologs. If the genes were not assigned names in *Drosophila* and honeybee (as in the case of the Hpx family), we followed the *Anopheles* classification (Waterhouse et al., 2007).

Sequence Alignment, Phylogenetic Analysis, and Domain Prediction

Multiple sequence alignments were initially made using the program ClustalX version 1.81 with default gap penalties (Thompson et al., 1997). In addition, multiple sequence alignments of some families were performed using “muscle,” a public domain protein multiple sequence alignment software (http://phylogenomics.berkeley.edu/cgi-bin/muscle/input_muscle.py). We manually trimmed the positions with a high percentage of

gaps (>70%). Phylogenetic trees were reconstructed from these alignments using the neighbor-joining method (Saitou and Nei, 1987) implemented in MEGA 4.0 program (Tamura et al., 2007). The pairwise deletion option was used in the NJ tree reconstruction and bootstrap support was evaluated based on 1,000 replicates. We also reconstructed the phylogenetic relationships of the *Cat* and *Hpx* genes by the program MrBayes v.3.1.2, which is a program based on Bayesian and maximum likelihood methods. The topologies of phylogenetic tree obtained by different approaches are similar. Thus, only the Bayesian tree is shown for respective antioxidant genes. All the domains of the putative insect *Hpx* genes are predicted online at http://smart.embl-heidelberg.de/.

Prediction of Subcellular Localization

Prediction of subcellular protein localization was performed for all identified antioxidant genes using the PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) program (Bannai et al., 2002). The sequences containing mitochondrial targeting peptides and signal peptides were then examined with the program TargetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000) and SignalP (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al., 2004) to look for signal/mitochondrial peptide cleavage sites and assess the likely subcellular localization of various gene products.

Expression Analysis with ESTs and Microarray Data

To search for transcriptional evidence of individual antioxidant genes, we used the putative coding sequences of the silkworm antioxidant genes as queries to perform blastn searches against the silkworm EST database. A 95% or greater identity and minimum cutoff E-value ($\leq 10^{-20}$) were employed to discriminate between duplicate genes. The detailed experimental process, quality control, consistency in replication for microarray data analysis were described in Xia et al. (2007). The names of probes for the silkworm antioxidant genes were listed in Table S1.

Gene Expression by RT-PCR

Total RNA was extracted from the fifth instar day 3 larvae tissues including testes, ovary, head, integument, fat body, midgut, haemocyte, Malpighian tubules, and silk glands using Trizol reagent (Invitrogen). The contaminating genomic DNA was digested with Rnase-free Dnase I (Promega) for 15 min at 37°C. The first strand of cDNA was synthesized using M-MLV reverse transcriptase following the manufacturer's instructions (Promega). RT-PCR primers were designed on the basis of the coding sequences of the silkworm antioxidant enzymes. As *Cat4*, *Cat5*, *Cat6*, and *Cat7* shared higher amino acid identities (>95%), we designed copy-specific primers based on the divergent DNA sequences to distinguish from different *Cat* genes (Table S2). The silkworm cytoplasmic actin A3 gene (forward primer: 5′-AACACCCCGTCCTGCTCACTG-3′; reverse primer: 5′-GGGCGAGACGTGTGATTTCCT-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 $MgCl_2$, 0.25 mM dNTP, 1× buffer and 2.5 units of Taq DNA polymerase (Promega). PCRs were performed with the following cycles: initial denaturation step of 95°C for 5 min, followed by 25 cycles of 95°C for 45 sec, 1 min annealing (temperatures listed in Table S2), 40 sec extension (72°C), and a final extension at 72°C for 10 min. The amplification products were analyzed on 1% agarose gels.

RESULTS

Some antioxidant genes have been identified in *D. melanogaster, An. gambiae,* and *Ap. mellifera* (Corona and Robinson, 2006). We used these known genes as queries to identify antioxidant genes in the *B. mori* and *T. castaneum* genomes. Fifty antioxidant genes were identified in the silkworm genome (Table 1 and Table S1). These identified genes include nine families SOD, CAT, GTPX, TPX, TRXR, TRX, GRX, MSR, and HPX. All of these families are major components of the antioxidant system. We found that SOD, GTPX, GRX, TRXR, MSR families were highly conserved in terms of phylogenetic orthologs, thus the gene numbers of the five families were almost the same in the five insects, especially, the 1:1:1:1:1 orthologous genes within these five families can be unambiguously defined. In addition, TPX family has been expanded independently in *D. melanogaster*, TRX family in *T. castaneum*, and HPX family in *An. gambiae*. Especially, Catalase (CAT) and heme-containing peroxidase (HPX) families also appear to be lineage-specifically expanded in the silkworm, which increased to six and seven members, respectively (Table 1). Thus, we mainly focus on these lineage-specifically expanded families in the silkworm.

Highly Conserved Families in Terms of Phylogenetic Orthologs in the Silkworm

Antioxidant and detoxification systems are crucial for insects to defense against xenobiotics, such as insecticides and pro-oxidant plant allelochemicals because these xenobiotics endogenously produce ROS in insects (Barbehenn and Stannard, 2004; Krishnan and Kodrik, 2006; Barbehenn et al., 2008). Thus, most families of insect antioxidant system are usually highly conserved in terms of phylogenetic orthologs, such as SOD, GTPX, GRX, TRXR, MSR families (Corona and Robinson, 2006). We also obtained similar results in this study. Compared with the *D. melanogaster*, *An. gambiae*, *T. castaneum*, and *Ap. mellifera* genomes, the silkworm genome has 6, 3, 5, 1, 2 genes, respectively, encoding SODs, GTPXs, GRXs, TRXRs, MSRs (Table 1 and Table S1). The numbers of these genes are roughly similar to those in other four insects, and most of them show obvious orthologous relationships (Figs. S1–S5). We found that the predicted proteins of these orthologous genes had higher amino acid identities and similar cell localizations and active sites (Figs. S1–S5). As described earlier, TPX family has been expanded independently in *D. melanogaster* and TRX family in *T. castaneum* (Table 1 and Figs. S6 and S7).

Expansions of Cat and Hpx Genes in the Silkworm

Expansion of CAT family. CAT is one of the antioxidant enzymes and usually breaks down H_2O_2 into O_2 and H_2O (Switala and Loewen, 2002). The phylogenetic tree of *Cats* in insects, mammals, bacteria, fungi, and plants was reconstructed, revealing seven clades: clades A (insect class), B (mammal class), C (silkworm class), D (nonsecreted bacterial class), E (plant class), F (secreted bacterial class), G (secreted fungal class) (Fig. 1). Strikingly, there is a conserved insect *Cat* cluster (clade A). The silkworm has not only a conserved *Cat* gene but also a lineage-specifically expanded *Cat* cluster with seven members (clade C). Interestingly, there are two divergent clusters of bacterial CATs, that is, the secreted bacterial CATs and nonsecreted bacterial CATs. In addition, *Cat* gene duplication also occurred in some insect lineages. For example, lineage-specific amplification of *Cat* was found in *Drosophila* and *T. castaneum*, corresponding to two and three duplicates, respectively.

Table 1. Major Components of the Enzymatic Antioxidant System of Ap. mellifera, D. melanogaster, An. gambiae, B. mori, and T. castaneum. NP Indicates Genes with No Automatic Prediction in Insects

Gene	*Apis mellifera*	*Anopheles gambiae*	*Drosophila melanogaster*	*Bombyx mori*	*Tribolium castaneum*
sod2	GB14346	AAR90328	CG8905	NP_001037299	Glean_05780
sod1	GB10133	XP_311594	CG11793	BAD69805	Glean_07011
sod3	NP	AAS17758	CG9027	BGIBMGA005489	Glean_11676
Ccs	GB14210	XP_308747	CG17753	BGIBMGA001698	Glean_10027
Rsod	GB14567	EAA00894	CG31028	BGIBMGA002311	GLean_11770
Sodq		EAA04552	CG5948	BGIBMGA002798	Glean_11675
Cat	GB11518	XP_314995	CG6871	NP_001036912	Glean_00084
			CG9314	BGIBMGA011431	Glean_11385
				BGIBMGA011430	Glean_11090
				BGIBMGA014411	
				BGIBMGA012691	
				BGIBMGA014510	
				BGIBMGA014284	
GtPx1	GB14138	XP_313166	CG12013	NP_001040104	Glean_10355
Gtpx2	GB18955	XP_562772		NP_001036999	Glean_10362
Gpx-like		XP_313167	CG15116	BGIBMGA013994	Glean_10354
Tpx1	GB19380	XP_308081	CG1633	NP_001037083	Glean_01700
Tpx2		XP_308336	CG1274	BGIBMGA002406	Glean_01071
Tpx3	GB10972	XP_565975	CG5826	NP_001040464	Glean_12328
Tpx4	GB10498	XP_320690	CG12405		Glean_04948
			CG11765		
			CG12896		
Tpx5	GB10803	XP_308753	CG3083		Glean_13791
Tpx6	GB15788		CG6888		Glean_14929
Trxr-1	GB14972	CAD30858	CG2151	BGIBMGA002818	Glean_07865
Trxr-2			CG11401		Glean_02698
Trx-1	GB17503	EAA04498	CG8993	NP_001040283	Glean_00058
			CG8517		
Trx-2	GB15855	EAA14495	CG31884	ABM_92269	Glean_15376
			CG3315		
Trx-3	GB19972	EAA09650	CG3719	BGIBMGA008199	Glean_07568
Trx1-like1	GB15457	EAA11972	CG5495	NP_001040348	Glean_09487
Trx1-like2	GB15572	XP_320264	CG14221	BGIBMGA006070	Glean_14394
			CG4193		
Trx1-like3	GB19276	XP_316887	CG9911	BGIBMGA006941	Glean_02136
					Glean_01643
Trxl-like4			CG13473		Glean_03523
					Glean_04980
					Glean_00309
					Glean_04373
					Glean_04723
Grx1	GB10598	XP_309539	CG6852	NP_001040246	Glean_05698
			CG7975	BGIBMGA008887	Glean_06382
Grx2	GB18700	XP_312440	CG14407	BGIBMGA008525	Glean_08371
Grx-like1	GB11664	EAA06446	CG31559	BGIBGA013430	Glean_07476
			CG12206		
Trx/Gtx	GB12870	EAA07378	CG6523	BGIBMGA006401	Glean_00304
MsrA	GB10196	XP_320164	CG7266	ABF_51258	Glean_05676
MsrB	GB15486	NP	CG6584	BGIBMGA007514	Glean_10923
Hpx1	GB10387	XP_311448	CG3477	BGIBMGA006520	Glean_00175
Hpx2	GB19819	XP_319784	CG8913		Glean_04551

Table 1. Continued

Gene	Apis mellifera	Anopheles gambiae	Drosophila melanogaster	Bombyx mori	Tribolium castaneum
Hpx3	GB11682	XP_313514	CG6879	BGIBMGA005680	Glean_15234
Hpx4	GB15860	XP_308561	CG12002	BGIBMGA000553	Glean_01556
Hpx5	XP_395479	XP_311106	CG5873	BGIBMGA014559	Glean_11222
Hpx6	GB13579	XP_309656	CG6969	BGIBMGA013482	Glean_04579
Hpx7		XP_309590		BGIBMGA012737	Glean_04592
Hpx8		XP_309592	CG7660	BGIBMGA013640	Glean_00751
Hpx9		XP_309791		BGIBMGA011886	
Hpx10		XP_309792		BGIBMGA012738	
Hpx11	GB19100	XP_309790		BGIBMGA012739	Glean_04661
Hpx12	GB18453	XP_300449		BGIBMGA012740	
Hpx13		XP_559246		BGIBMGA000238	
HPX14		ENSANGP00000001781		BGIBMGA000457	
HPX15		XP_554228	CG4009	BGIBMGA000239	
HPX16		XP_309429		BGIBMGA006519	
DBLOX	GB12650	XP_317106	CG10211	BGIBMGA007042	Glean_05493
DUOX	GB13459	XP_311449	CG3131	BGIBMGA005478	Glean_02498

In cluster A of Figure 1, the silkworm CAT Bm-NP_001036912 shared 67.2% of amino acid identity with the honeybee CAT Am-GB11518, 70.5% with the fruit fly CAT Dm-CG6871, 67.8% with the red flour beetle CAT Tc-Glean_00084, 60.9% with the fruit fly CAT Dm-CG9314, and 34.8% with the mosquito CAT Ag-XP_314995. All genes encoding these proteins were phylogenetically related and had the peroxysomal targeting sequences (PTS). Moreover, we found that BmCAT3, BmCAT4, BmCAT5, BmCAT6, and BmCAT7 had a signal peptide, respectively, and transmembrane domains except for BmCAT4. Since presence of signal peptide and absence of transmembrane domains can be used as indicators of a secreted protein (DebRoy et al., 2010), then BmCAT4 might be a putative secreted protein. In addition, *BmCat1*, *BmCat2*, *BmCat3*, *BmCat5* had one intron, whereas *BmCat4*, *BmCat6*, *BmCat7* had two introns (Fig. S8).

To confirm the structure of *BmCat4* gene, we searched the silkworm EST dataset downloaded from GenBank using its putative coding sequence as queries. We obtained 74 ESTs of Verson's gland and assembled these ESTs into a contig. Strikingly, this contig had polyadenylation signals near their 3′ terminal end and no Shine-Delgarno ribosome-binding site upstream of the start codon (Fig. S9). Furthermore, we sequenced the genomic region of *BmCat4* gene and the obtained sequence is consistent with the corresponding region of the genome sequence. The alignment of the genomic sequence and cDNA contig of *BmCat4* showed that this gene had two introns (Fig. S9). In addition, *BmCat* genes and bacterial *Cat* genes formed distinct clades in the phylogenetic tree (Fig. 1), the putative secreted BmCAT4 is not a result of contamination of bacterial DNAs in genome sequencing. All these evidence suggested that *BmCat4* is a typical eukaryotic nuclear gene and BmCAT4 might be a secreted protein.

Duplicated silkworm CATs might be composed of several functionally overlapping and compensatory components that protect silkworm from endogenous and exogenous oxidative stresses. Similarity analysis of amino acid sequences indicated that the protein Bm-NP_001036912 shared 34.7–44.7% amino acid identities with other BmCATs. As shown in Figure 2, Bm-NP_001036912 and Dm-CG6871 contained 36 residues interacting with a heme cofactor (Murthy et al., 1981), BmCAT4, BmCAT5, and DmCG9314 contained

Figure 1. The Bayesian tree of CAT amino acid sequences of insect, fungi, bacteria, plant, and mammal. Numbers beside nodes indicate the supporting values measured by the posterior probabilities. There are seven distinct clades of catalases: clade A, insect CATs; clade B, mammal CATs; clade C, *B. mori* CATs; clade D, non-secreted bacterial CATs; clade E, plant CATs; clade F, secreted bacterial CATs; clade G, secreted fungal CATs. Bm, *B. mori.* Ag, *An. gambiae.* Am, *Ap. mellifera.* Dm, *D. melanogaster.* Tc, *T. castaneum.* Hs, *Homo sapiens.* Pa, *Pongo abelii.* Mm, *Macaca mulatta.* Cj, *Callithrix jacchus.* Ame, *Ailuropoda melanoleuca.* Clf, *Canis lupus familiaris.* Pp, *Photobacterium profundum.* Hp, *Haenophilus parasuis.* Ms, *Methylocella silvestris.* Ss, *Sphingobacterium spiritivorum.* Sg, *Sodalis glossinidius.* Zm, *Zymomonas mobilis.* Aa, *Alicyclobacillus acidocaldarius.* Bs, *Bacillus* sp. Kr, *Kteclonobacter racemifer.* Bj, *Brassica juncea.* Rs, *Raphanus stivus.* Bn, *Brassica napus.* Gh, *Gossypium hirsutum.* Pd, *Populus deltoids.* Hp, *Hypericum perforatum.* At, *Arabidopsis thaliana.* Pa, *Podospora anserina.* Nc, *Neurospora crassa.* Cp, *Claviceps purpurea.* Mo, *Magnaporthe oryzae.* Bg, *Blumeria graminis.* Bf, *Botryotinia fuckeliana.* Bu, *Burkholderia ubonensis.* Bo, *Burkholderia oklahomensis.* Bg, *Burkholderia glumae.* Pf, *Pseudomonas fluorescens.* Ks, *Klebsiella* sp. Kp, *Klebsiella pneumonia.* Mc, *Methylobacterium chloromethanicum.* Mr, *Methylobacterium radiotolerans.* Me, *Methylobacterium extorquens.* Mp, *Methylobacterium populi.* Bx, *Burkholderia xenovorans.* Xo, *Xanthomonas oryzae.* Xc, *Xanthomonas campestris.* The putative CAT family members identified in *B. mori* are marked with large black dots.

21 residues interacting with a heme cofactor, BmCAT2 and BmCAT3 had 17 residues interacting with a heme cofactor. Other insect CATs all contained more than 21 residues interacting with a heme cofactor (Fig. 1 and Fig. S10). A previous study suggested that the enzyme DmCG9314 lacked 15 of the 36 heme-binding residues and probably had

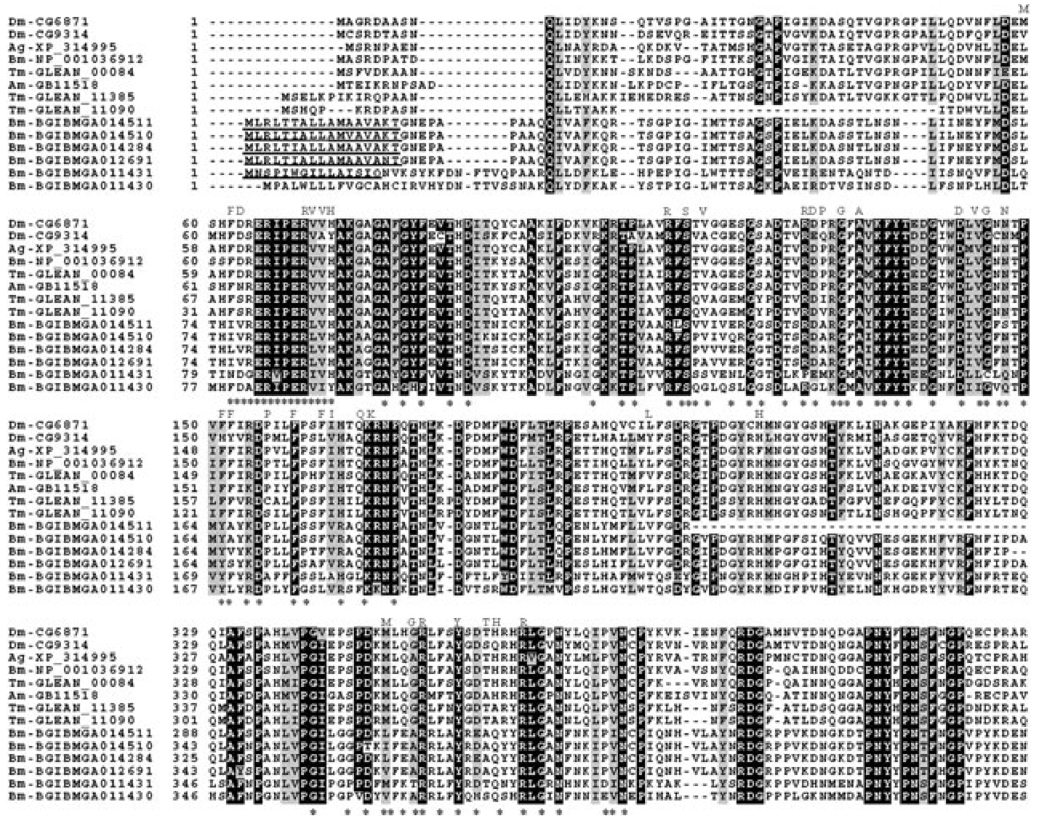

Figure 2. The alignment of amino acid sequences of *Cat* genes in *B. mori* and other insects. Identical residues are shaded black, while similar residues are gray. NADPH-binding residues are marked with asterisks. The residues interacting with a heme group is indicated above alignment. Predicted signal peptide for BGIBMGA014511, BGIBMGA014510, BGIBMGA014284, BGIBMGA012691, and BGIBMGA011431 are underlined.

no activity of converting H_2O_2 into H_2O and O_2 (Waterhouse et al., 2007). We found that BmCAT2, BmCAT3, BmCAT4, and BmCAT5 also lacked 15 or more than 15 heme-binding residues. Thus, these enzymes might have lost the activity of converting H_2O_2 into H_2O and O_2.

Duplication of Hpx family in silkworm. HPX can use H_2O_2 as an electron acceptor to catalyze oxidation of a variety of substrates, playing important roles in innate immunity, synthesis of thyroid hormone, as well as in pathogenesis of a number of inflammatory diseases in mammals (Cheng et al., 2008). A double peroxidase (DBLPX) is one of the insect HPXs that combine a peroxidase with two highly divergent N-terminal (DBLPX-N) and C-terminal (DBLPX-C) peroxidase domains. Dual oxidase is another HPX, which contains a NADPH-oxidase domain. HPX family has five members in vertebrates (Waterhouse et al., 2007), whereas this family was significantly amplified in insects, especially in the silkworm (17 members) and mosquito *An. gambiae* (18 members). Insect HPX family can be divided into 11 clades and contains two confident 1:1:1:1:1 orthologous clades (G and J) in the phylogenetic tree. Domain architectures analysis suggested that HPX has four major classes, including peroxinectin, peroxidasin, DUOX, and DBLPX (Fig. 3A).

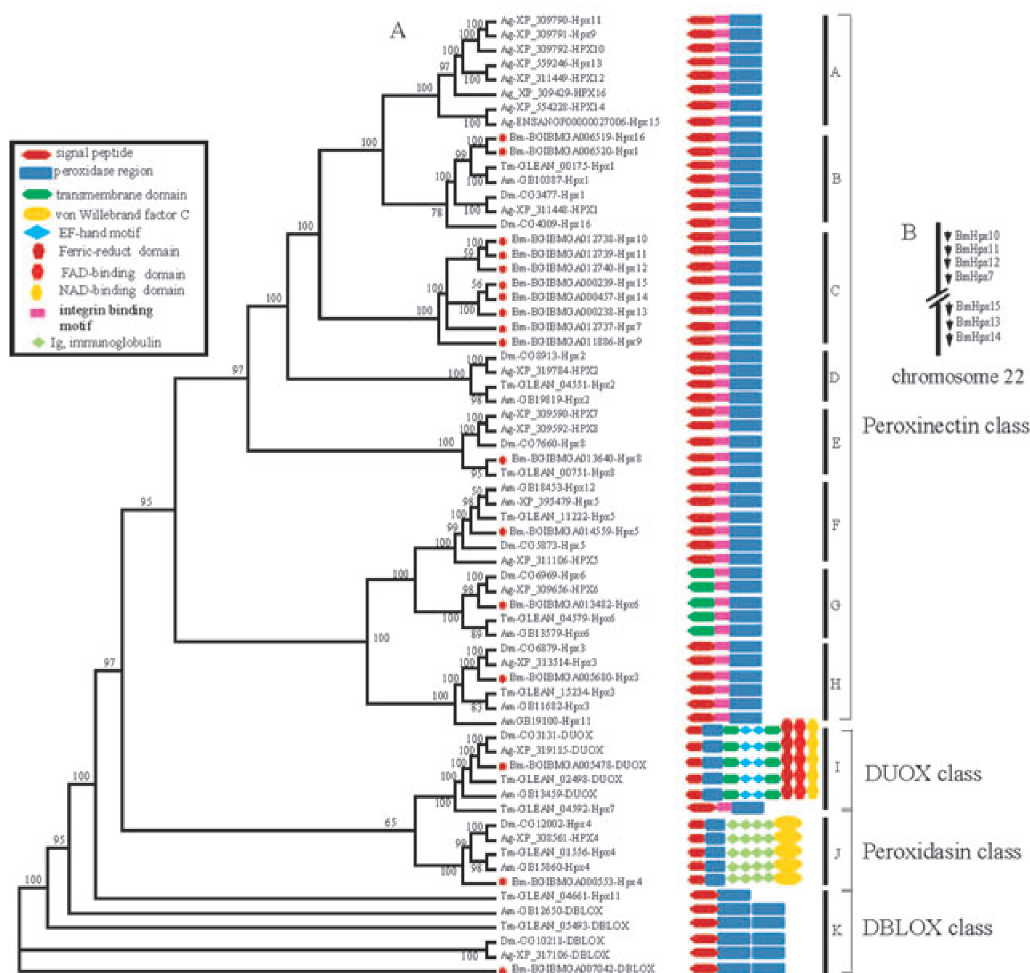

Figure 3. The Bayesian tree of insects and the cluster organization of *BmHpx* genes. (a) The Bayesian tree of the heme-containing peroxidases protein family in insects. Numbers beside nodes indicate the supporting values measured by the posterior probabilities. The putative HPX family members identified in *B. mori* are marked with large red dots. The domain architectures are listed in the black frame on the right. (b) The cluster organization of *Hpx* genes in *B. mori* genome. Only those genes involved in clusters of two or more *Hpx* genes on the same scaffolds are shown. The arrows indicate orientation of each gene.

In peroxinectin class, the proteins contain a signal peptide, a putative integrin-binding motif (Lys-Gly-Asp, KGD) and the peroxidase domain, except for the proteins of the clade G. It can be found from clades A and C that the silkworm HPX *BmPxts* and mosquito HPX *AgPxts* obviously experienced lineage-specific expansions, with eight and seven members, respectively. In addition, the silkworm *BmHpx7*, *BmHpx10*, *BmHpx11*, and *BmHpx12* were tandem arranged genes, while *BmHpx13*, *BmHpx14*, and *BmHpx15* genes were also tandem arranged. All of tandem arranged genes were located on chromosome 22 and had the same transcription direction (Fig. 3B). The silkworm BmHPX1 and other insects' HPX1s showed relatively high amino acid identities (27.6–40.2%), whereas the silkworm BmHPX16 and other insects' HPX1s showed relatively low amino acid identities (20.9–34.8%). Also, the fruit fly DmHPX1 showed higher amino acid identities (24–62.8%)

than DmHPX16 with other insects' HPX1s (20.9–36%). Therefore, Bm/Dm HPX1 and the three other insects' HPX1s may be orthologs. The range of amino acid identities among the red flour beetle TcHPX2 and three other insects' HPXs was larger than that of TcHPX11 with three other insects' HPXs in clade D and the corresponding silkworm orthologous genes might have been lost. In clade E, *Hpx* gene was lost in *Ap. mellifera*, while duplicated in *An. gambiae* (*AgHpx7* and *AgHpx8*). The mosquito AgHPX8 and three other insects' HPXs showed higher amino acid identities (32.5–41.3%) than the AgHPX7 and three other insects' HPXs (30.7–32.5%). The honeybee AmHPX5 and four other insects' HPX5s showed higher amino acid identities (19–36.2%) than the AmHPX12 and four other insects' HPX5s (3.8–20.1%). Thus, *AmHpx5* and four other insects' *Hpx* genes may be orthologs. In clade H, the silkworm *BmHpx3* and four other insects' *Hpx3* genes may be orthologs, and the honeybee *AmHpx11* is phylogenetically related to this group.

In DUOX class, *DUOX* genes in *D. melanogaster*, *An. gambiae*, *Ap. mellifera*, silkworm, and *T. castaneum* showed orthologous relationship, while duplicated in *T. castaneum* (Fig. 3A). The primary translation product of *DUOX* consists of a C-terminal signal peptide, a peroxidase domain, two EF-hand domains between transmembrane domains, the ferric-reduct domain, and FAD-binding domain followed by NAD-binding domain. Similar to DUOX class, *DBLOX* genes in the five insects also showed orthologous relationship and duplicated in *T. castaneum* (in clade K of Fig. 3A). The primary translation product of *DBLOX* contains a C-terminal signal peptide followed by N-terminal (DBLPX-N) and C-terminal (DBLPX-C) peroxidase domains (Fig. 3A). In addition, peroxidasin genes in the five insects showed confident 1:1:1:1:1 orthologous relationship (in clade J of Fig. 3A). The primary translation products of peroxidasin genes contain a C-terminal signal peptide, followed by four immunoglobulin-like domains, the peroxidase domain, and a carboxy terminal Willebrand type C protein–protein interaction domain.

Expression of silkworm antioxidant genes. In order to detect the expression of the silkworm antioxidant genes, we searched the silkworm EST dataset downloaded from GenBank using the putative coding sequences as queries. We found that 42 of the 50 antioxidant genes have ESTs and most of the transcriptionally active genes were expressed in a tissue-specific manner. Based on the tissue expression microarray, the probes of 39 antioxidant genes were found and only 24 of which showed expression signals with values more than 400 at least in one tissue. The clustering analysis suggested that the expressed antioxidant genes were classified into four groups (I–IV) and most of them showed similar expression levels between male and female except for group I (Fig. 4A). In group I, there were five genes predominantly expressed in testis. In group II, there were six genes predominantly expressed in the head, the fat body, and the Malpighian tubule. In group III, *BmSod3* and *BmTrx3* had relatively high expression levels in the midgut and the Malpighian tubule. In addition, *BmTpx2* also showed higher expression level in the midgut. The silk gland specific group includes six genes; they belong to *Hpx*, *Trx*, and *RSod* genes, respectively (Fig. 4A IV). However, *BmHpx13* and *BmHpx14* were also expressed in the head of females. In total, most of the *Sods*, *Trxs*, and *Tpxs* were predominantly expressed in the testis, the midgut, and the Malpighian tubule, whereas *Hpxs* were predominantly expressed in the head, the integument, the fat body, and the silk gland.

As for those genes without microarray probes, RT-PCR was performed to analyze their tissue expression patterns in the fifth instar day 3 larvae. We found that 18 of the 25 antioxidant genes were transcribed (Fig. 4B). Among them only *Gpxs*, *Grxs*, and *Msrs* showed high expression levels in various tissues, thus these genes might play housekeeping roles in the silkworm. All of other genes showed tissue-specific expression patterns. For

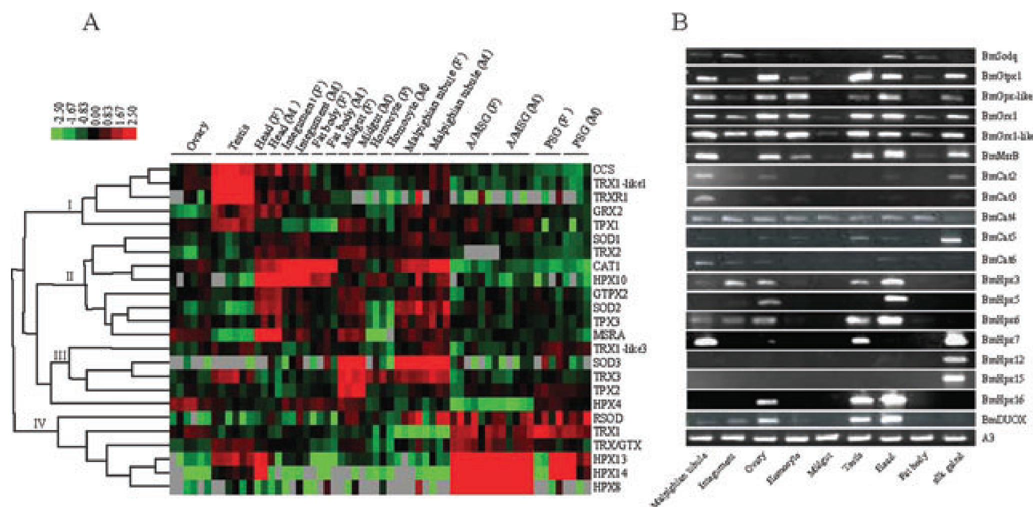

Figure 4. Tissue expression patterns of the silkworm antioxidant genes in different larvae tissues based on microarray data and RT-PCR. (a) Expression patterns for silkworm antioxidant genes in different tissues of 3-day fifth larvae by microarray analysis. Red color represents positive; black color represents zero; green color represents negative; gray color represents missing. (b) Tissue expression patterns of the silkworm antioxidant genes that did not show expression signals in microarray data in multiple tissues on day 3 the 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.

example, *BmHpx12* and *BmHpx15* were only expressed in silk gland and the six genes, *BmCat2/Cat3/Cat4/Cat6*, *BmHpx6*, and *BmHpx7*, were predominantly expressed in the Malpighian tubule. In addition, *BmCat2, BmHpx6*, and *BmHpx7* were also expressed in other tissues, such as the ovary, the silk gland, the integument, the ovary, the testes, and the head. *BmHpx6* and *BmHpx3* had very similar expression patterns. Moreover, *BmHpx16* and *BmDUOX* had relatively high expression levels in the ovary, the testes, and the head. In addition, *BmSodq* was predominantly expressed in the integument and the head.

DISCUSSION

We have identified the 50 antioxidant genes belonging to nine genes families based on the new assembly of the silkworm genome (Table 1). The phylogenetic analyses showed that most of antioxidant genes had confident orthologs in the five insects investigated. However, the silkworm CAT and HPX families experienced lineage-specific expansions, which increased to six and seven members, respectively. Furthermore, some antioxidant gene families duplicated in other insects. For instance, TPX family was expanded in *D. melanogaster*; TRX family in *T. castaneum*; HPX family in *An. gambiae*. This indicated that gene duplication may play important roles for insects to adapt to different habitats.

Insect *Grx* genes also duplicated in *An. gambiae*, *D. melanogaster*, *Ap. mellifera*, *B. mori*, and *T. castaneum*, corresponding to 4, 6, 4, 5, and 5 duplicates, respectively. There are two groups of 1:1:1:1:1 orthologs (Fig. S3, clades A and B), suggesting that some *Grx* genes duplicated before the radiation of the five species. *BmGrx2* and its corresponding orthologs shared high amino acid identities and all of them have signal peptides for transporting into mitochondria. In addition, insect GRX2 contains C-G-F-S active domains, thus they

belong to monothiol GRX. All of the *Grx* genes might be initially derived from dithiolic type and the origin of CPYC class Grxs was much earlier than the CGFS class Grxs (Alves et al., 2009). Thus, insect *Grx2* (CGFS) probably originated from *Grx1* (CPYC). However, *BmGrx1* and its corresponding orthologs shared low amino acid identities, suggesting that insect *Grx1* genes might have experienced rapid divergence.

Although insect *Gpx* genes do not show an obvious orthologous relationship, at least one of the two paralogs in each insect contains signal peptide, implying that they may have extracellular functions. In mammals, it was assumed that *Gpx* gene family might originate from a common ancient gene (Gpx4/PHGpx), and then gene duplications occurred and followed by random integration of the genome (Brigelius-Flohe et al., 1994; Dufaure et al., 1996). The ancestral *PHGpx* duplicated and the duplicated genes were divided into two groups: one is the genes that encode cellular GPX1 and GPX2 proteins, and the other is the genes that encode the secreted GPX3, GPX5, and GPX6 proteins (Herbette et al., 2007). Insect GPXs might experience similar evolutionary processes and then were divided into intracellular and secreted forms. For example, Bm/AmGPX2s are putatively located extracellular while Bm/AmGPX1s intracellular. Cysteine involved in the regeneration of non-Se-GPX by TRX as an electron donor is not strictly conserved in all non-Se-GPXs (Dayer et al., 2008). Bm/AgGPX1s belong to non-Se-GPXs and do not have this cysteine (Fig. S2). In addition, the lack of Se-dependent glutathione peroxidases (GPXs) in insects increases potential importance of the putative Se-independent peroxidase function of GSTs in antioxidant defense. Thus, the GSTs might have the function of the GPXs in antioxidant defense of insects. Since the silkworm GST genes have been investigated at genome scale in a previous study (Yu et al., 2008), they were not included in the present study.

Cat genes were expanded in the silkworm. The silkworm *Bmcat1* (NP_001036912) shared the highest amino acid identities with its orthologous genes compared with other *Bmcat* genes. Thus, we assumed that *Bmcat1* might be the ancient one and other *Bmcat* genes might be duplicated from *Bmcat1*. In the silkworm, four of the *Bmcat* genes lost their 15 or more residues interacting with a heme cofactor, they might lack the activities of converting H_2O_2 into H_2O and O_2 (Waterhouse et al., 2007). However, these genes also had ESTs and were expressed in the fifth instar day 3 larvae (Fig. 4B). The similar case was also found in *D. melanogaster* (Waterhouse et al., 2007). Whether these *Bmcat* genes that lost some residues interacting with a heme cofactor have evolved novel functions needs further experimental supports. Indeed, *cat* genes might have other new functions, such as oxidase activity (Vetrano et al., 2005) and hydrogen-transfer activity (Gaetani et al., 2005).

It should be pointed out that BmCAT4 could be a secreted protein based on its predicted subcellular location and amino acid sequence characteristics. However, further sequence analysis indicated that BmCAT4 may lose the activity of converting H_2O_2 into H_2O and O_2. Moreover, BmCat4 appears to be ubiquitously expressed including hemocytes (except in silk gland). As no secreted CAT has been found in insects so far, it is likely that *Bmcat4* might gain new functions and it is interesting to determine functions of *Bmcat4* and another three *Bmcat* genes that lost some residues interacting with a heme cofactor in future.

Peroxinectin class of the *Hpx* genes was obviously expanded in the silkworm. In contrast, other classes of the *Hpx* genes already lost some members (Fig. 3). This suggested that new *Hpx* genes were created probably as a result of consecutive gene duplication, whereas some *Hpx* genes might be deleted. This implies that a birth-and-death evolutionary mechanism may make HPX family maintain diverse members (Nei and Rooney, 2005;

Zamocky et al., 2008). The expanded peroxinectin genes of the silkworm were tandem arranged on two regions of chromosome 22 (Fig. 3B). Probably, these genes might be produced by local duplications. The similar case was also found in *An. gambae* (Fig. 3, clade A). The expanded *AnPxts* were highly expressed in the salivary glands and their encoding enzymes held the activity of peroxidases. These enzymes could destroy hemostatically active amines released by vertebrate hosts (Ribeiro and Nussenzveig, 1993; Ribeiro et al., 1994). In addition, invertebrate peroxinectins were also described to possess the peroxidase activity and cell-adhesion activity by proteolytical processing after their release from the cell (Soderhall, 1999). For the expanded *BmPxt* genes, they were predominantly expressed in silk gland of silkworm based on the microarray data and RT-PCR analysis (Fig. 4A, group IV and Fig. 4B). Thus, we predicted that BmPXTs as antioxidant could maintain cell homeostasis in the process of the synthesis of relatively large amounts of silk proteins.

It can be seen from the micoarray data and RT-PCR results of fifth instar day 3 larval tissues that the silkworm antioxidant genes showed different expression patterns. This indicated that these antioxidant genes might have different physiological functions. One cluster in group (I) including five antixodant genes was found to be predominantly expressed in the testis. Sperm longevity could be increased by antioxidative enzymes which can reduce ROS levels, such as SOD and TPX (Collins et al., 2004). Thus, high expression level of the genes in testis (Fig. 4A, group I) may be related to the longevity of the silkworm sperm. Previous studies suggested that *Hpx* and *Cat* genes might participate in innate immune responses of insects (Waterhouse et al., 2007; Zou et al., 2007). Since integument is one of immune tissues (Waterhouse et al., 2007; Zou et al., 2007), an observation that the *BmHpx10* and *BmCat1* genes were highly expressed in integument (Fig. 4A, group II) suggested that these genes might be related to immunity. The midgut is the primary barrier to the allelochemicals ingested, *Sod* and *Tpx* can be induced by allelochemical, such as quercetin and glycosides (Krishnan and Kodrik, 2006; Krishnan and Sehnal, 2006; Chaudhary et al., 2007). Indeed, mulberry leaves contain these secondary metabolites. Therefore, the high expression of *Sod3*, *Trx3*, and *Tpx2* in the midgut suggested that these genes might play important roles in tolerating the related allelochemicals (Fig. 4A, group III). All of the *BmCats* were transcribed except for *BmCat7* and most of them showed low expression level in multiple tissues in RT-PCR (Fig. 4B). Furthermore, *Cat2/Cat3/Cat4/Cat6* were predominantly expressed in the Malpighian tubule (Fig. 4B). Insect Malpighian tubule has detoxification function similar to mammalian kidney (Dow, 2009). Thus, we speculate that those *BmCat* genes predominantly expressed in the Malpighian tubule may play important roles in detoxification of related superoxides and allelochemicals (Giovanini et al., 2006). In addition, some ROS are produced in the process of the synthesis of relatively large amounts of silk proteins. Therefore, the relatively high expression of *BmCat2* and *BmCat5* in silk gland suggested that the *BmCats* might be essential to protect silk gland from oxidative damage (Fig. 4B). Furthermore, the *BmSods*, *BmTrxs*, *BmHpxs*, *BmMsrs*, *BmGpxs*, *BmGrxs*, and *BmTpxs* showed high constitutive expression levels in various tissues, they might play the housekeeping role in reducing oxidative stress damages.

ACKNOWLEDGMENTS

We would like to thank Dr. Lei Shi in our lab for critical reading of the manuscript.

LITERATURE CITED

Alves R, Vilaprinyo E, Sorribas A, Herrero E. 2009. Evolution based on domain combinations: the case of glutaredoxins. BMC Evol Biol 9:66.

Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S. 2002. Extensive feature detection of N-terminal protein sorting signals. Bioinformatics 18(2):298–305.

Barbehenn RV, Stannard J. 2004. Antioxidant defense of the midgut epithelium by the peritrophic envelope in caterpillars. J Insect Physiol 50(9):783–790.

Barbehenn RV, Maben RE, Knoester JJ. 2008. Linking phenolic oxidation in the midgut lumen with oxidative stress in the midgut tissues of a tree-feeding caterpillar *Malacosoma disstria* (Lepidoptera: Lasiocampidae). Environ Entomol 37(5):1113–1118.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S. 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340(4):783–795.

Brigelius-Flohe R, Aumann KD, Blocker H, Gross G, Kiess M, Kloppel KD, Maiorino M, Roveri A, Schuckelt R, Usani F, et al. 1994. Phospholipid-hydroperoxide glutathione peroxidase. Genomic DNA, cDNA, and deduced amino acid sequence. J Biol Chem 269(10):7342–7348.

Chaudhary A, Pechan T, Willett KL. 2007. Differential protein expression of peroxiredoxin I and II by benzo(a)pyrene and quercetin treatment in 22Rv1 and PrEC prostate cell lines. Toxicol Appl Pharmacol 220(2):197–210.

Cheng G, Salerno JC, Cao Z, Pagano PJ, Lambeth JD. 2008. Identification and characterization of VPO1, a new animal heme-containing peroxidase. Free Radic Biol Med 45(12):1682–1694.

Cho YH, Lee EJ, Roe JH. 2000. A developmentally regulated catalase required for proper differentiation and osmoprotection of *Streptomyces coelicolor*. Mol Microbiol 35(1):150–160.

Collins AM, Williams V, Evans JD. 2004. Sperm storage and antioxidative enzyme expression in the honeybee, *Apis mellifera*. Insect Mol Biol 13(2):141–146.

Corona M, Robinson GE. 2006. Genes of the antioxidant system of the honeybee: annotation and phylogeny. Insect Mol Biol 15(5):687–701.

Dayer R, Fischer BB, Eggen RI, Lemaire SD. 2008. The peroxiredoxin and glutathione peroxidase families in *Chlamydomonas reinhardtii*. Genetics 179(1):41–57.

DebRoy S, Keenan AB, Ueno N, Jeronimo SM, Donelson JE, Wilson ME. 2010. Leishmania infantum chagasi: a genome-based approach to identification of excreted/secreted proteins. Exp Parasitol 126(4):582–591.

Dow JA. 2009. Insights into the Malpighian tubule from functional genomics. J Exp Biol 212(Part 3):435–445.

Duan J, Li R, Cheng D, Fan W, Zha X, Cheng T, Wu Y, Wang J, Mita K, Xiang Z, Xia Q. 2010. SilkDB v2.0: a platform for silkworm (*Bombyx mori*) genome biology. Nucleic Acids Res 38 (Database issue):D453–D456.

Dufaure JP, Lareyre JJ, Schwaab V, Mattei MG, Drevet JR. 1996. Structural organization, chromosomal localization, expression and phylogenetic evaluation of mouse glutathione peroxidase encoding genes. C R Acad Sci III 319(7):559–568.

Emanuelsson O, Nielsen H, Brunak S, von Heijne G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300(4):1005–1016.

Gaetani GF, Ferraris AM, Sanna P, Kirkman HN. 2005. A novel NADPH: (bound) NADP+ reductase and NADH: (bound) NADP+ transhydrogenase function in bovine liver catalase. Biochem J 385(Part 3):763–768.

Giovanini MP, Puthoff DP, Nemacheck JA, Mittapalli O, Saltzmann KD, Ohm HW, Shukle RH, Williams CE. 2006. Gene-for-gene defense of wheat against the Hessian fly lacks a classical oxidative burst. Mol Plant Microbe Interact 19(9):1023–1033.

Herbette S, Roeckel-Drevet P, Drevet JR. 2007. Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. FEBS J 274(9):2163–2180.

Hermes-Lima M, Zenteno-Savin T. 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp Biochem Physiol C Toxicol Pharmacol 133(4):537–556.

Kanzok SM, Fechner A, Bauer H, Ulschmid JK, Muller HM, Botella-Munoz J, Schneuwly S, Schirmer R, Becker K. 2001. Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*. Science 291(5504):643–646.

Kim YJ, Lee KS, Kim BY, Choo YM, Sohn HD, Jin BR. 2007. Thioredoxin from the silkworm, *Bombyx mori*: cDNA sequence, expression, and functional characterization. Comp Biochem Physiol B Biochem Mol Biol 147(3):574–581.

Krishnan N, Kodrik D. 2006. Antioxidant enzymes in *Spodoptera littoralis* (Boisduval): are they enhanced to protect gut tissues during oxidative stress? J Insect Physiol 52(1):11–20.

Krishnan N, Sehnal F. 2006. Compartmentalization of oxidative stress and antioxidant defense in the larval gut of *Spodoptera littoralis*. Arch Insect Biochem Physiol 63(1):1–10.

Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G, Kafatos FC, Barillas-Mury C. 2003. The role of reactive oxygen species on Plasmodium melanotic encapsulation in *Anopheles gambiae*. Proc Natl Acad Sci USA 100(24):14139–14144.

Lee KS, Kim SR, Park NS, Kim I, Kang PD, Sohn BH, Choi KH, Kang SW, Je YH, Lee SM, Sohn HD, Jin BR. 2005. Characterization of a silkworm thioredoxin peroxidase that is induced by external temperature stimulus and viral infection. Insect Biochem Mol Biol 35(1):73–84.

Li X, Schuler MA, Berenbaum MR. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol 52:231–253.

Murthy MR, Reid TJ, Sicignano A, Tanaka N, Rossmann MG. 1981. Structure of beef liver catalase. J Mol Biol 152(2):465–499.

Nei M, Rooney AP. 2005. Concerted and birth-and-death evolution of multigene families. Annu Rev Genet 39:121–152.

Radyuk SN, Sohal RS, Orr WC. 2003. Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and under-expression of thioredoxin peroxidase in Drosophila cells. Biochem J 371(Pt 3):743–752.

Ribeiro JM, Nussenzveig RH. 1993. The salivary catechol oxidase/peroxidase activities of the mosquito *Anopheles albimanus*. J Exp Biol 179:273–287.

Ribeiro JM, Nussenzveig RH, Tortorella G. 1994. Salivary vasodilators of Aedes triseriatus and *Anopheles gambiae* (Diptera: Culicidae). J Med Entomol 31(5):747–753.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–425.

Soderhall K. 1999. Invertebrate immunity. Dev Comp Immunol 23(4-5):263–266.

Switala J, Loewen PC. 2002. Diversity of properties among catalases. Arch Biochem Biophys 401(2):145–154.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24(8):1596–1599.

The International Silkworm Genome Consortium. 2008. The genome of a lepidopteran model insect, the silkworm *Bombyx mori*. Insect Biochem Mol Biol 38(12):1036–1045.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25(24):4876–4882.

Vetrano AM, Heck DE, Mariano TM, Mishin V, Laskin DL, Laskin JD. 2005. Characterization of the oxidase activity in mammalian catalase. J Biol Chem 280(42):35372–35381.

Wang Q, Chen K, Yao Q, Zhao Y, Li Y, Shen H, Mu R. 2008. Identification and characterization 18 of a novel 1-Cys peroxiredoxin from silkworm, *Bombyx mori*. Comp Biochem Physiol B Biochem Mol Biol 149(1):176–182.

Wang Y, Oberley LW, Murhammer DW. 2001. Antioxidant defense systems of two lipidopteran insect cell lines. Free Radic Biol Med 30(11):1254–1262.

Waterhouse RM, Kriventseva EV, Meister S, Xi Z, Alvarez KS, Bartholomay LC, Barillas-Mury C, Bian G, Blandin S, Christensen BM, Dong Y, Jiang H, Kanost MR, Koutsos AC, Levashina EA, Li J, Ligoxygakis P, Maccallum RM, Mayhew GF, Mendes A, Michel K, Osta MA, Paskewitz S, Shin SW, Vlachou D, Wang L, Wei W, Zheng L, Zou Z, Severson DW, Raikhel AS, Kafatos FC, Dimopoulos G, Zdobnov EM, Christophides GK. 2007. Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. Science 316(5832):1738–1743.

Xia Q, Cheng D, Duan J, Wang G, Cheng T, Zha X, Liu C, Zhao P, Dai F, Zhang Z, He N, Zhang L, Xiang Z. 2007. Microarray-based gene expression profiles in multiple tissues of the domesticated silkworm, *Bombyx mori*. Genome Biol 8(8):R162.

Yamamoto K, Banno Y, Fujii H, Miake F, Kashige N, Aso Y. 2005a. Catalase from the silkworm, *Bombyx mori*: gene sequence, distribution, and overexpression. Insect Biochem Mol Biol 35(4):277–283.

Yamamoto K, Zhang P, Banno Y, Fujii H, Miake F, Kashige N, Aso Y. 2005b. Superoxide dismutase from the silkworm, *Bombyx mori*: sequence, distribution, and overexpression. Biosci Biotechnol Biochem 69(3):507–514.

Yamamoto K, Zhang P, He N, Wang Y, Aso Y, Banno Y, Fujii H. 2005c. Molecular and biochemical characterization of manganese-containing superoxide dismutase from the silkworm, *Bombyx mori*. Comp Biochem Physiol B Biochem Mol Biol 142(4):403–409.

Yu Q, Lu C, Li B, Fang S, Zuo W, Dai F, Zhang Z, Xiang Z. 2008. Identification, genomic organization and expression pattern of glutathione S-transferase in the silkworm, *Bombyx mori*. Insect Biochem Mol Biol 38(12):1158–1164.

Zamocky M, Jakopitsch C, Furtmuller PG, Dunand C, Obinger C. 2008. The peroxidase-cyclooxygenase superfamily: reconstructed evolution of critical enzymes of the innate immune system. Proteins 72(2):589–605.

Zhao L, Shi L. 2009. Metabolism of hydrogen peroxide in univoltine and polyvoltine strains of silkworm (*Bombyx mori*). Comp Biochem Physiol B Biochem Mol Biol 152(4):339–345.

Zhao LC, Shi LG. 2010. Metabolism of hydrogen peroxide between diapause and non-diapause eggs of the silkworm, *Bombyx mori* during chilling at 5 degrees C. Arch Insect Biochem Physiol 74(2):127–134.

Zou Z, Evans JD, Lu Z, Zhao P, Williams M, Sumathipala N, Hetru C, Hultmark D, Jiang H. 2007. Comparative genomic analysis of the *Tribolium* immune system. Genome Biol 8(8):R177.