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Comparison of gloverin gene expression patterns between domesticated and wild silkworms

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Abstract

Bombyx mandarina is widely accepted as ancestor of *B. mori*. Silkworms are served as well-characterized models for understanding the mechanism for the genetic regulation of development. In this study, we performed RNA-Seq analysis to examine tissue-expression of gloverin isoforms of the silk-gland, mid-gut, and fat body in *B. mandarina*. BLAST analysis revealed that four gloverin isoform gene sequences of *B. mandarina* were highly similar to *B. mori.* To identify the difference between two species, the expression profile of gloverin was measured by semi- RT-PCR analysis. The specific expression of gloverin isoform genes was observed mainly in the fat body from *B. mori* but not *B. mandarina*. However, all of tissues in the wild-type silkworm could induce the upregulation of compared with the *B. mori*. To validate the sudden increase in gloverin gene expression in the mid-gut tissue of *B. mandarina*, we were using qRT-PCR. Relative mRNA expression rate of gloverin at the wild-type silkworm was much higher than domestic silkworm. Comparative genomics between domesticated and wild silkworms showed different tissue-expression levels in some of immune related genes. These results are suggesting a trend toward decreasing immunity related genes expression during domestication. Further studies are needed to elucidate the silkworm domestication and an invaluable resource for wild silkworm genomics research.

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Introduction

A characteristic of insect immunity is the rapid and transient activation of immune genes to produce effectors in response to microbial infection (Yang *et al.,* 2015). Antimicrobial peptides (AMPs) are an important factor in insect immune response, to combat invading pathogens such as bacteria, fungi, and viruses (Hoffman *et al.,* 1999; Hoffman, 2003; Hultmark, 2003; Kaneko *et al.,* 2007; Kurata *et al.,* 2006; Lemaitre, 2004). Insect AMPs are synthesized and regulated by the Toll and immune deficiency (IMD) pathways in the fat body, hemocytes, and other tissues (Bulet *et al.,* 1999; Lemaitre and Hoffmann, 2007; Yang *et al.,* 2015). Several AMPs, cecropin, moricin, attacin and lebocin, which have a broad spectrum of antimicrobial activities, have been identified from the silkworm, *B. mori* (Hara and Yamakawa, 1995a; Hara and Yamakawa, 1995b; Kaneko *et al.,* 2007).

Gloverin, which is one of well-studied AMPs in

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lepidopteran insect immunity, was first reported from the giant silk moth, *Hyalophora gloveri* (Axe´n *et al.,* 1997). This peptide revealed antibacterial activity exclusively against Gram-negative bacteria, showing in many respects activity similar to attacin (Kaneko *et al.,* 2007). In *B. mori*, four *gloverin* genes (Bmglv1–4) have been identified (GeneBank accession numbers AB190863, AB190864, Ab190865, and AB190866), and Bmglv2–4 are derived from duplicating Bmglv1 (Kaneko *et al.,* 2007; Kawaoka *et al.,* 2008; Mrinal and Nagaraju, 2008; Yang *et al.,* 2015).

The *B. mori* Microarray Database (BmMDB) provides information for tissue-specific gene expression by using the whole-genome oligonucleotide microarray in the silkworm (Park *et al.,* 2010). Huge numbers of transcriptomes were analyzed at the whole-genome level by means of microarray. Whole-genome mRNA sequencing (RNA-Seq) technologies have been a significant advance for high-throughput transcriptome analyses, as they can generate hundreds of millions reads in a single sequencing run (Castillo *et al.,* 2015; Ozsolak and Milos, 2011; Wang *et al.,* 2009). This is more sensitive, quantitative and efficient, and it has higher reproducibility compared to previously use hybridization-based microarray techniques (Castillo *et al.,* 2015; de Klerk *et al.,* 2014). RNA-Seq has already produced exciting and novel information in the study of various diseases (Costa *et al.,* 2013; Castillo *et al.,* 2015; Xuan *et al.,* 2013). This powerful tool is becoming increasingly attractive for investigating the transcriptional profiles in model and non-model organisms (Castillo *et al.,* 2015; Daines *et al.,* 2011; Ekblom and Galindo, 2011). The silkworm genome contains about 14,623 genes and larvae multiple tissues transcriptional data were obtained using a 22,987 oligonucleotide probe microarray (Huang *et al.,* 2009; Xia *et al.,* 2004; Xia *et al.,* 2008). The genome-wide analysis of model insects showed that the numbers of immune related genes in *A. gamibae* and *D. melanogaster* are greater than those in *B. mori* and *A. mellifera*, but their innate immune signal transduction pathways are rather primitive (Huang *et al.,* 2009; Tanaka *et al.,* 2008; Weinstock *et al.,* 2006).

In this study, we used RNA-Seq. analysis to examine tissue-expression of immune related genes in the wild-type silkworm, *B. mandarina*, and were analyzed for the tissue-specific expression pattern of these genes. To identify the difference between two species, *B. mori* (domesticated) and *B. mandarina* (wild-type), the expression profile of gloverin was measured by semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis. Subsequently, we analyzed the mRNA expression levels of the *gloverin* gene in *B. mori* and *B. mandarina* by quantitative real-time RT-PCR (qRT-PCR) to validate specific expression patterns. In particular, the relative mRNA expression of *gloverin* gene was highest in mid-gut in *B. mandarina* than *B. mori*. To the best of our knowledge, this is the first report of a mid-gut specific expression of the *gloverin* gene in *B. mandarina*.

Material and Methods

Experimental silkworm strain

The fifth instar larvae of *B. mori* strain, jam 125, was preserved from the National Academy of Agricultural Science (Wanju, Korea), and reared on fresh mulberry leaves under standard conditions (25~26 °C and 65~75 % relative humidity). Samples of *B. mandarina* larva were collected from its natural environment (Suwon, Korea), and bred in cultivation plastic box with the softwood cutting of mulberry tree at 25~26 °C and 65~75 % humidity.

Tissue collection and RNA preparation

We dissected and collected mid-gut, silk gland, blood and fat body tissues from a single fifth-instar larva of *B. mandarina* and *B. mori*, respectively. Tissues were immediately frozen and stored in liquid nitrogen. Total RNAs were extracted using TRIzol Reagent (Life Technologies, Frederick, Maryland, USA) according to the manufacturer's instructions. The quality and quantity of extracted total RNAs were tested using Agilent Bioanalyzer 2100 (Agilent Technologies) and nanophotometer (Implen, Germany). The total RNAs were stored at -70 °C until further use.

RNA sequencing and data analysis

Sequencing libraries were constructed using Illumina TruSeq RNA preparation kits following the manufacturer's instructions. Libraries were checked and quantified using Agilent Bioanalyzer 2100 (Agilent Technologies) and Qubit dsDNA BR Assay Kit

(Invitrogen, http://products. invitrogen.com). Libraries were sequenced using an Illumina HiSeq 2000 platform, and 100 bp paired-end reads were generated. Data analysis and base calling were performed by Illumina instrument software. Mapping of the paired-end read sequenced data were conducted using Bowtie2 software based on silkworm genomic database, (SilkDB: http://www.silkdb.org/silkdb/). In order to examine the tissue specific expression of immune related genes in the *B. mandarina*, the mapping conducted depth was sorted out considering the frequency analysis after adjusting normalization value that is read per kilobase of exon model per million mapped reads (RPKM) value (Mortazavi *et al*. 2008). The annotation of genes was performed by BLAST in SilkDB (http://www.silkdb.org/silkdb/).

Reverse transcription PCR (RT-PCR)

Total RNAs were treated with DN*ase* I (Life Technologies) for 15 min at 37°C to remove genomic DNA. Oligo dT-primed cDNA was synthesized from total RNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster city, CA, USA). Reverse transcription was performed for 2 h at 37°C. To detect the target genes, RT-PCR was performed using Top-*Taq* PreMix polymerase (CoreBioSystems, Korea). Initial amplification using specific primers (Table 1) was performed under the following conditions: denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, primer extension at 72°C for 1 min 20 s, and a final extension at 72°C for 5 min. The reaction mixture was stored at 4°C. The amplified PCR products were separated using electrophoresis on 1% agarose gels, respectively.

Table 1. List of oligonucleotide sequence for RT-PCR

Genes	Primers
BmA3	Forward: TTGGCATCATACCTTCTACA
	Reverse: ATCTACGCCATTGCTGAAGC
Gloverin 1	Forward: TATCTTCGCTACAACTCTGGTG
	Reverse: GGTGTCGCCTTGAGGATTG
Gloverin 2	Forward: ACGCCAAGGCTGCTATTG
	Reverse: GCTGACAAGTGAGTGTTCTTAC
Gloverin 3	Forward: ACAGAACGACGATGGACTATTC
	Reverse: AGACCCTGGTGCCGTAAG
Gloverin 4	Forward: GAATGCGGAAGCTGCTATTG
	Reverse: GAGGCGGGTGTTCTTGTC
Lebocin 3	Forward: CTTCGTGCCAGAGGTTCATC
	Reverse: GATACAGCCATAGCGGTTCC

Quantitative real-time PCR (qRT-PCR)

We conducted qRT-PCR in a 20 μL reaction mixture containing 10 μL of SYBR Premix Ex Taq (Takara, Japan) according to the manufacturer's instructions, using a specific primer set (Table 1). PCR reactions were programmed as follows: 40 cycles of denaturation at 95°C for 5 s and annealing and elongation at 60°C for 35 s. Fluorescence was detected at the end of every 60°C extension phase. To quantify the amount of target mRNAs in *B. mori* and *B. mandarina*, the *Actin* gene was used as an internal reference to normalize the quantity of total RNA purified from insects of different tissues (e.g., *B. mori* and *B. mandarina*). Real-time PCR was performed using a SYBR Green PCR kit and the ABI7300 real-time PCR instrument (Applied Biosystems, CA, USA). Changes in gene expression levels were determined using the comparative C_T method as described in the ABI Prism 7700 Sequence Detection System manual (User Bulletin #2 from Applied Biosystems).

Results

RNA seq. analysis and gene annotation

RNAs from the four tissues, mid-gut, silk gland, blood and fat body, of *B. mandarina* were sequenced using an Illumina HiSeq 2000 platform. After base calling, 11.68 Gb of the clean reads were obtained for the four tissues transcriptomes (Table 2). The clean reads were mapped to the *B. mori* reference genomic database (SilkDB: http://www.silkdb.org/silkdb/) and gene annotation using Bowtie2 software. The uniquely mapped read were normalized and calculated using the reads per kilobase per million reads (RPKM) method as the following formula to estimate the abundance of transcripts of each immune related genes.

$RPKM = 10^9 C/NL$.

Where C is the number of read uniquely mapped to the interest gene, N is the number of reads uniquely mapped to the total

Table 2. Distribution of transcript levels in the four different wide silkworm tissues and summary of the sequence assembly after Illumina sequencing

Sample Name	RPKM Interval (%)		Raw reads	Clean reads	Clean bases (GB)	Error rate (%)
Silk glands	0 ~ 1	7,211(48.4)	38,244,387	38,213,357	2.90	0.08
	1 ~ 3	1,926(12.9)				
	3 ~ 15	3,524(23.7)				
	15 ~ 60	1,405(9.4)				
	>60	827(5.6)				
Mid-gut	0 ~ 1	5,998(41.0)	38,143,113	38,111,691	2.90	0.08
	1 ~ 3	1,468(10.0)				
	3 ~ 15	3,067(21.0)				
	15 ~ 60	2,384(16.3)				
	>60	1,706(11.7)				
Fat body	0 ~ 1	7,812(53.4)	35,265,696	35,236,346	2.68	0.08
	1 ~ 3	2,081(14.2)				
	3 ~ 15	2,970(20.3)				
	15 ~ 60	1,112(7.6)				
	>60	648(4.4)				
Blood	0 ~ 1	5,749(39.3)	42,093,164	42,059,124	3.20	0.08
	1 ~ 3	1,112(7.6)				
	3 ~ 15	3,034(20.7)				
	15 ~ 60	3,073(21.0)				
	>60	1,655(11.3)				

transcripts. L is the total length of exons from the interest gene. With the use of adjusted RPKM, the frequency analysis was carried out using the 2 x 3 contingency Table 2, which illustrates the number of mapped reads for each sample and interest gene, and the difference between total number of reads and number of mapped reads. Cut off was determined at the level of 10^{-6}, based on the Bonferroni correction, considering the number of total transcriptome is 12,411.

Transcriptome analysis of immune related genes in *B. mandarina* by NGS analysis

A total 1,642 tissue-specific genes were differentially expressed (Bonferroni 10^{-7}) between four different tissues (Data not shown). We found 300 gene isoforms upregulated in the four different tissues (silk gland : 54, fat body : 6, mid-gut : 216, and blood : 24, respectively). As shown in Table 3, quantitative RNA-Seq analysis revealed the tissue-dependent differential expression of immune related genes. Mid-gut, fat body, and blood tissues presented with expressed antitrypsin isoform 2, gloverin, paralytic peptide binding protein, peptidoglycan recognition protein, and phenoloxidase subunit at higher levels than silk gland (Table 3). In the mid-gut and fat body tissues, five antibacterial peptide genes encoding gloverin 1 ~ 4 and lebocin 3, were significantly specifically expressed. These results suggest that some of the differentially expressed immune-related genes might be important in the regulation of immune function against the pathogens.

Expression analysis of four *gloverin* genes in *B. mandarina* and *B. mori*

An initial control RT-PCR was performed using the internal control primers to test for genomic DNA contamination in the RNA isolated from three different tissues and to assess the quality of the synthesized cDNA. We confirmed that the isolated

Table 3. A comparison of the *B. mandarina* immune related genes expression patterns by RNA-Seq. analysis.

Gene name	microArray (BmMDB)	CDS ID (SilkDB)	RPKM			
			gland	midGut	fat	blood
lysozyme	sw13847	BGIBMGA010439-TA	3.757	780.646	5062.328	43.302
antitrypsin isoform 2	sw11925	BGIBMGA009953-TA	4.787	242.221	3937.763	11667.64
gloverin1	sw01773	BGIBMGA013863-TA	0.644	764.204	2016.225	25.567
gloverin2	sw12523	BGIBMGA005658-TA	3.145	5542.689	2696.629	105.373
gloverin3	sw22043	BGIBMGA013803-TA	0.331	1380.508	1069.178	22.113
gloverin4	-	BGIBMGA013865-TA	1.842	3283.519	979.155	278.444
lebocin-3	sw08844	BGIBMGA006775-TA	0.96	1789.971	1598.657	343.638
paralytic peptide	-	BGIBMGA014551-TA	0.873	40.456	1329.886	357.521
paralytic peptide binding protein 1	sw22741	BGIBMGA010168-TA	1.186	1.88	1.551	6626.906
beta-1,3-glucan-bindingprotein	sw21934	BGIBMGA011608-TA	0.277	640.719	732.159	570.125
peptidoglycan recognition protein	sw22599	BGIBMGA012864-TA	0.317	1382.084	0.298	36.837
peptidoglycan-recognition protein 2	sw08563	BGIBMGA000583-TA	0.472	2130.374	1.689	23.797
peptidoglycan recognition protein S6	sw20314	BGIBMGA012866-TA	0.67	1727.539	203.649	50.616
peptidoglycan recognition protein S2	sw21727	BGIBMGA007987-TA	1.763	4684.766	50.073	61.124
phenoloxidase subunit 1	sw19955	BGIBMGA012763-TA	0.42	1.447	1.146	3486.363
phenoloxidase subunit 2	sw05533	BGIBMGA013115-TA	1.826	10.95	3.985	10645.6

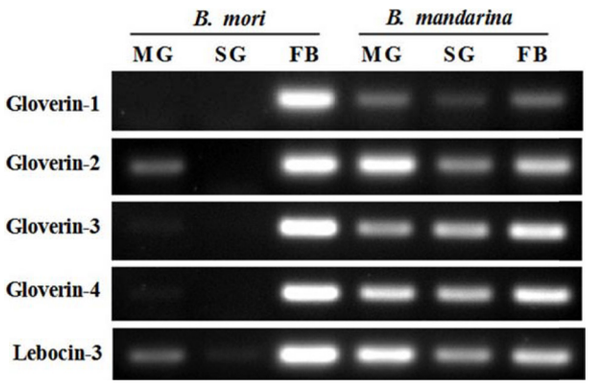

Fig. 1. RT-PCR analysis of four gloverin isoforms and lebocin 3 in *B. mandarina* and *B. mori.* A control PCR was performed using the internal control primers and cDNA from both species. Analysis of four gloverin *and* lebocin gene expression in the two species shows the different expression patterns. MG; mid-gut, SG; silk gland, FB; fat body.

RNA contained no genomic DNA and that the synthesized cDNA was suitable for RT-PCR analysis of the target genes. RT-PCR was used to examine target gene expression in total RNAs isolated from different tissue of *B. mandarina* and *B. mori*. The expression profile of four *gloverin* genes in the three different tissues of *B. mori* and *B. mandarina* is shown in Fig. 1. Four *gloverin* genes were expressed in fat body tissue of both silkworms. Interestingly, all of tissues in the *B. mandarina* could induce the upregulation of compared with the *B. mori*.

To determine the levels of four gloverin mRNA expression according to different tissues (mid-gut, silk glands, and fat body) and silkworms (domestic and wild-type), the qRT-PCR method was used. The expression of target genes was normalized to the levels of the silkworm *Actin* gene, which was utilized as an internal reference. The C_T values obtained indicated differential expression depending on the different tissues and silkworm. Specifically, we determined the relative expression of gloverin mRNA from three different tissues in both domestic and wild-type silkworms (Fig. 2). Interestingly, gloverin mRNA expression was lower in the fat body than the mid-gut issue of *B. mandarina*. And also, the expression level increased again in the mid-gut tissue of *B. mandarina* and reached the highest level compare to *B. mori*. To determine why there was a sudden increase in *gloverin* expression in the mid-gut tissue of *B. mandarina*, the levels of *gloverin* expression in the mid-gut tissue of both silkworms were analyzed by qRT-PCR (Fig. 3). Surprisingly, mRNA expression of *gloverin* at the wild-type silkworm was much higher than domestic silkworm. This result expounds that the increased expression of *gloverin* in the mid-gut tissue of *B. mandarina* may have been caused that *gloverin* gene was participating in *B. mandarina* immunity to live in the wild.

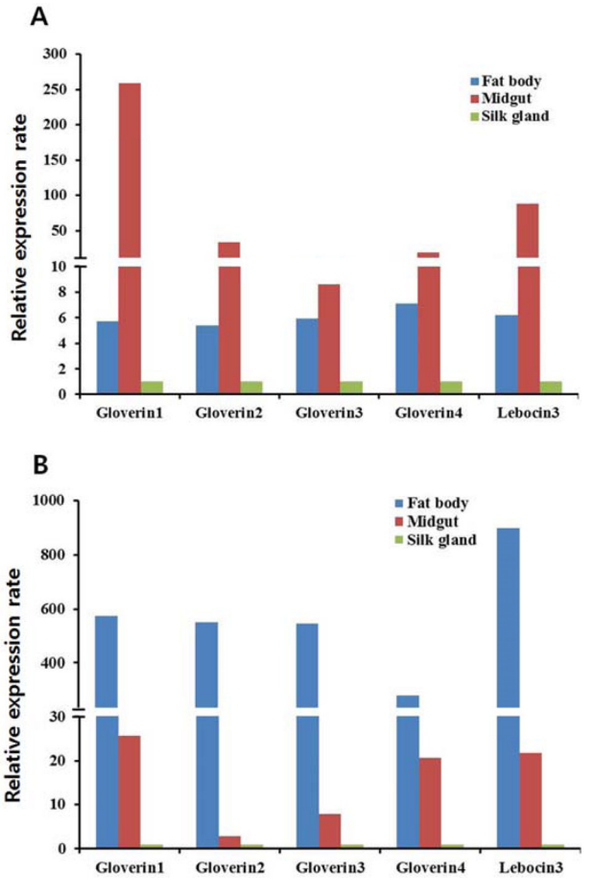

Fig. 2. Quantitative real-time RT-PCR analysis of four gloverin isoforms and lebocin 3 in three different tissues of *B. mandarina* (A) and *B. mori* (B). The expression of genes were normalized to the expression levels of the silkworm *Actin* gene as an internal reference. The Y-axis indicates the relative transcription level. MG; mid-gut, SG; silk gland, FB; fat body.

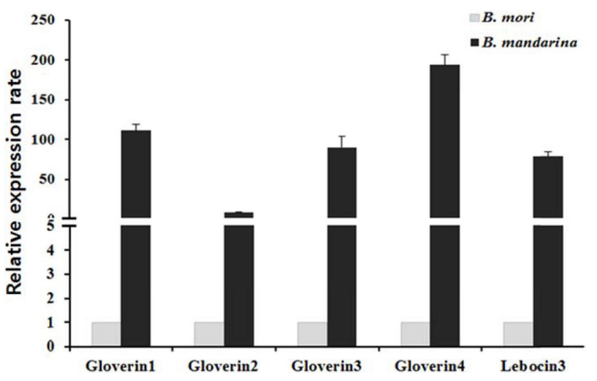

Fig. 3. Relative expression of four gloverin isoforms and lebocin 3 in the mid-gut tissue of both silkworms. The expression of genes were normalized to the expression levels of the silkworm *Actin* gene as an internal reference. The transcription level of gloverin isoforms and lebocin of *B. mandarina* were compared with the rates in *B. mori*.

Discussion

The wild-type silkworm *B. mandarina* belongs to Lepidoptera *Bombycidae* (Cheng *et al.,* 2015). It is widely accepted as that *B. mandarina* is widely accepted as an ancestor of the domesticated silkworm, *B. mori* (Cheng *et al.,* 2015). *B. mandarina* have been domesticated for at least 5,000 years to increase and improve cocoon yield (Cheng *et al.,* 2015). Recently, genome-wide transcriptional analysis of silkworm larvae have found that identified significant upregulation or downregulation of several immune genes (Huang *et al.,* 2009; Kaneko *et al.,* 2007; Yang *et al.,* 2015). This technique has also been used as an efficient method to study adaptation to high-elevation environments or domestication (Cheng *et al.,* 2015). Based on *de novo* assembled transcripts and identification of orthologous genes, nonsynonymous site/synonymous site (Ka/Ks) analysis can provide insights into the process of adaptive evolution or domestication (Cheng *et al.,* 2015; Liu *et al.,* 2014; Szovenyi *et al.,* 2015). Recently, expression profile analysis found significant differential expression between the domestic and wild-type silkworms. Based on NGS results, some studies have found that phenotypic changes are involved in genetic divergences (Cheng *et al.,* 2015). For example, copy number variation (CNV) is also an important to understand domestication mechanism in silkworms. About 319 CNVs have been identified and are mainly related to reproduction, immunity, detoxification and signal recognition (Cheng *et al.,* 2015; Zhao *et al.,* 2014). The copy number of carotenoid-binding protein (CBP) gene varies from 1 to 20 among *B. mori* strains. In contrast to *B. mori*, *B. mandarina* was found to possess a single copy of CBP lacking a retrotransposon insertion, regardless of habitat (Cheng *et al.,* 2015).

In this study, we performed RNA-Seq for *B. mandarina* using Illumina HiSeq2000 sequencing platform. The BmMDB provides information for tissue-specific gene expression by using the whole-genome oligonucleotide microarray in the *B. mori* (Park *et al.,* 2010; Xia *et al.,* 2007). We generated a number of unigenes. By comparison between domesticated and wild-type silkworm genes, we identified orthologous genes and analyzed the tissue-specific expression patterns of four *gloverin* genes in the silk gland, fat body, and mid-gut five days of fifth instar larvae during the development of both silkworms. BLAST analysis revealed that the nucleotide sequences of expressed four *gloverin* genes in

B. mandarina were highly similar to *B. mori*. These results show that four *gloverin* genes are evolutionarily conserved in the lepidopteran lineage (Table 2). Semi-quantitative RT-PCR was used to examine target gene expression from different tissue of *B. mandarina* and *B. mori*. As shown in Fig. 1, four *gloverin* genes were mainly expressed in fat body tissue of domesticated silkworm. However, all of tissues in the wild-type silkworm could induce the upregulation of compared with the *B. mori*. To determine the relative expression of gloverin mRNA during three different tissues in both domesticated and wild-type silkworms we performed qRT-PCR. Interestingly, gloverin mRNA expression was slightly higher in the mid-gut than the fat body tissue of *B. mandarina* (Fig. 2), and reached the more high level compare to mid-gut tissue of *B. mori* (Fig. 3). As shown results, mRNA expression of *gloverin* at the wild-type silkworm was much higher than domestic silkworm. The domestication mechanism may involve regulatory elements. Comparative genomics between domesticated and wild silkworms showed different insect defense systems as AMPs in domesticated silkworms as in their wild counterparts, suggesting a trend toward decreasing immunityrelated genes expression during domestication. Genes that are immunityrelated and have low expression in domesticated silkworms have experienced selective sweep, indicating a possible correlation with indoor feeding environment and the enlargement of silk glands in domesticated silkworms (Cheng *et al.,* 2015; Xiang *et al.,* 2013).

This result expounds that the increased expression of *gloverin* in the mid-gut issue of *B. mandarina* may have been caused that *gloverin* gene was participating in *B. mandarina* immunity to live in the wild. Functional analysis of these genes provided information about the domestication mechanism in silkworms (Cheng *et al.,* 2015). In addition to genetic divergence, epigenetic divergence is also important in silkworm domestication. Further studies are needed to elucidate the silkworm domestication and an invaluable resource for wild silkworm genomics research.

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