Research paper

RNA-seq profiles of putative genes involved in specific immune priming in *Bombyx mori* haemocytes

Yunhong Yi[a], Hui Xu[b], Mei Li[c,*], Gongqing Wu[a,*]

[a] *School of Chemistry and Chemical Engineering, Guangdong Pharmaceutical University, Zhongshan 528458, China*
[b] *Jingzhou Academy of Agricultural Sciences, Jingzhou 434000, China*
[c] *University of Electronic Science and Technology of China, Zhongshan Institute, Zhongshan 528402, China*

ARTICLE INFO

ABSTRACT

Background: The immune system of many invertebrates, including insects, has been shown to comprise memory, or specific immune priming. However, knowledge of the molecular mechanisms especially the candidate immune-related genes mediated the specificity of the immune priming are still very scarce and fragmentary. We therefore used two closely related Gram-negative pathogenic bacteria (*Photorhabdus luminescens* TT01 and *P. luminescens H06*) as the priming agents and employed Illumina/Solexa platform to investigate the transcriptional changes of the haemocytes of *Bombyx mori* larvae after priming.
Results: In total, 23.0 Gbp of sequence data and 153,331,564 reads were generated, representing 10,496 genes. Approximately 89% of the genes or sequenced reads could be aligned to the silkworm reference genome. The differentially expressed genes (DEGs) of PBS-vs-TT01 (up-regulated expression of TT01 relative to PBS), PBS-vs-H06 (up-regulated expression of H06 relative to PBS) and TT01-vs-H06 (up-regulated expression of H06 relative to TT01) were 707, 159 and 461 respectively. In addition, expression patterns of 25 selected DEGs derived from quantitative real-time polymerase chain reaction (qRT-PCR) were consistent with their transcript abundance changes obtained by transcriptomic analyses. The DEGs are mainly related to pattern recognition receptors (PRRs), antimicrobial peptides (AMPs), signaling molecular, effector molecules, phagosome and spliceosome, indicating that they have participated in the regulation of the specific immune priming in the *B. mori* larvae.
Conclusions: The transcriptome profiling data sets from this study will provide valuable resources to better understand the molecular and biological mechanisms regulating the specificity of invertebrates' immune priming. All these will shed light on controlling insect pests or preventing epidemic of infectious diseases in economic invertebrates.

1. Introduction

Immune responses are historically divided into two categories, adaptive and innate. Adaptive immune response is characterized as recombination-activating gene (RAG)-dependent immunity that involves somatic recombination in T and B lymphocytes. Invertebrates have always been considered to possess only innate immune, because they lack the lymphocytes and immunoglobulin. Typically, innate immune responses are not characterized as possessing specificity and memory. However, recent studies have demonstrated that invertebrates possess a memory-like response, a phenomenon that has been termed "immune priming" (Galvez and Chapuisat 2014; Hernandez Lopez et al., 2014; Moreau et al., 2012; Tidbury et al., 2011). And also various other terms have been used to describe the similar phenomena, such as "responsive mode prophylaxis" (Moret and Siva-Jothy, 2003), "tolerance" (Kurtz and Armitage, 2006), "trained immunity" (Netea et al., 2011) and "potentiation" (Boraschi and Italiani, 2018b). Whatever this phenomenon is called, prior exposure to a pathogen endows the hosts an enhanced immune protection is the typical characteristic of the memory-like response.

Immune priming has been demonstrated in a large number of insects, such as *Galleria mellonella* (Wu et al., 2014), *Manduca sexta* (Eleftherianos et al., 2006) and *Anopheles gambiae* (Rodrigues, 2010), although it is a controversial issue in many other studies (Boraschi and Italiani, 2018a; Cooper and Eleftherianos, 2017; Reber and Chapuisat, 2012). The controversy on immune priming main comes from the different species of the host or the pathogens used for challenge. For example, recent study showed that immune priming in ant queens varies according to mating status and mating conditions or species (Galvez and Chapuisat, 2014). Moreover, we found that there was typical immune priming in the *G. mellonella* primed with an appropriate dose of heat-killed bacteria *Photorhabdus luminescens* TT01, *Bacillus thuringiensis* HD-1 or lipopolysaccharide (LPS). We also found that the extent of enhanced immune protection in *G. mellonella* depended on the time elapsed between the priming and challenge, whereas no evidence for priming response has been found in *G. mellonella* larvae exposed to toxin protein PirA2B2 from *P. luminescens* TT01 (Wu et al., 2015a; Wu et al., 2015c; Wu et al., 2014). All of these studies suggested that insect immune priming is a complex process.

The degree of specificity in immune priming is varied among different insect species, and even associated with the timing of priming and infection, and the elicitors that were used for priming. Specificity here defines the degree to which a primed immune response is able to discriminate among different pathogens. Evidence for a range of specificities in immune priming, including coarse specific and highly specific responses, has been found in *American cockroach* (Faulhaber and Karp, 1992), *Bombus terrestris* (Sadd and Schmid-Hempel, 2006), *Tribolium castaneum* (Roth et al., 2009) and *Drosophila melanogaster* (Pham et al., 2007). Our recent study demonstrated that *B. mori* larvae can even discriminate the bacteria at strain level, indicating a degree of specificity exists in the larval priming response, and this specific priming response was positive correlated to the phagocytic ability of the hemocytes (Wu et al., 2015b). However, the molecular mechanisms especially the putative genes involved in mediating the specific immune priming are still unknown.

The *B. mori* larvae with a fully-sequenced genome are not only of important economic value, but is also a model organism for studies of invertebrate immunity. In order to fight against invading pathogens, silkworm developed three types of mechanisms. One is the humoral immune responses including secretion of antimicrobial peptides (AMPs) into the hemolymph through the Toll, Imd and JAK/STAT pathway (Hua et al., 2016; Taniai et al., 1996). The second is the haemocyte-mediated cellular immune responses, such as clearing pathogenic microorganisms by phagocytosis or nodulation and killing large intruder by encapsulation (Wago, 1983). The third is an enzymatic cascade leading to melanisation (Li et al., 2016).

Next-generation sequencing (NGS), also called high-throughput or deep sequencing technology which contain Illumina Solexa, Roche 454, and ABI SOLiD platforms have revolutionized genomics because they allow faster and less expensive sequencing (Morozova and Marra, 2008). NGS has been successfully applied in the following research areas, such as resequencing, small RNA expression, DNA methylation, SSR mining, SNP discovery and de novo transcriptome (RNA-Seq) of non-model organisms. Recently, next-generation high-throughput sequencing techniques, such as Illumina sequencing and 454 Pyrosequencing, have also been employed to investigate the genetic background and immune response of insects, such as *Meligethes aeneus* (Vogel et al., 2014), *Protaetia brevitarsis seulensis* (Bang et al., 2015), *Plutella xylostella* (Etebari et al., 2011), *Nilaparvata lugens* (Bao et al., 2012) *Frankliniella occidentalis* (Zhang et al., 2013) and *Cimex lectularius* (Bai et al., 2011).

In this study, we injected the *B. mori* larvae with two closely related Gram-negative pathogenic bacteria (*Photorhabdus luminescens* TT01 and *P. luminescens* H06) and examined the molecular mechanism mediated the specificity of immune-priming from transcriptomic views using NGS technology. Three transcriptomes of the immune-primed (TT01 and

H06) and non-primed *B. mori* larvae (PBS) were generated and compared. We identified differentially expressed genes related to specific immune priming and verified the precision of the transcriptome results using quantitative real-time PCR (qRT-PCR). We discuss the regulatory mechanism of the differentially expressed genes (DEGs) involved in specific immune priming. Some significant pathways, such as phagosome and endocytosis signaling pathway were also analyzed. The data obtained in this study will provide a valuable resource for future genetic and genomic research on invertebrate immunity.

2. Materials and methods

2.1. Insects, bacteria and immune-priming

The *Bombyx mori* strain p50 (DaZao) larvae were reared on mulberry leaves at 25 °C with a 12 h light and a 12 h dark cycle. The one-day-old fifth-instar *B. mori* larvae were selected for the experiments. The bacteria *Photorhabdus luminescens* TT01 and *P. luminescens* H06 were prepared according to our previous study and used for immune-priming (Wu et al., 2015b).

For immune-priming, the *B. mori* larvae were divided into three groups (20 larvae per group), two of the groups were immune-primed with 10 μl of the PBS solution injected into the hemocoel that contained 1×10^5 heat-killed cells of TT01 or H06 per larva. The third group was injected with 10 μl of the PBS solution and was used as the control. After injection, the larvae of each group were reared individually with diet at 25 °C. Forty-eight hours after the immune-priming, about 50 μl of hemolymph was collected from each larva of each group and preserved in Trizol in liquid nitrogen for RNA isolation. For all treatments, three independent trials were performed.

2.2. RNA extraction

The total RNA was isolated using the Trizol Kit (Promega, USA) and then treated with RNase-free DNase I (Takara Bio, Japan) for 30 min at 37 °C to remove residual DNA. The quality of RNA was verified using Agilent Technologies 2100 Bioanalyzer System and the RNA integrity was determined via RNase free agarose gel electrophoresis. Only those RNA samples whose 260 nm/280 nm ratio was between 1.8 and 2.0 were used for subsequent analyses.

2.3. RNA-Seq library construction and sequencing

We pooled RNA samples for the same treatments of the three independent biological replicates at an equal ratio and then the pooled RNA were used for the cDNA synthesis. Poly (A) mRNA was isolated using oligo-dT beads (Qiagen). All mRNA was broken into short fragments (~300 nt) by adding fragmentation buffer. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by the synthesis of the second-strand cDNA using RNase H and DNA polymerase I. The cDNA fragments were purified using a QIAquick PCR purification kit and then washed with EB buffer for end reparation poly (A) addition. After that, the short fragments were ligated with sequencing adapters. Following agarose gel electrophoresis, the cDNA fragments (~300 bp) were purified and enriched by PCR to construct the final cDNA library. The cDNA library was sequenced on the Illumina sequencing platform (Illumina HiSeq™ 2500) using the paired-end technology by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.4. Illumina reads processing and annotation of gene expression levels

Low quality reads were filtered by excluding those reads containing adaptors, reads with > 5% unknown nucleotides, and low-quality reads with > 50% of bases with a quality value ≤5. The resulting high quality reads were mapped to ribosome RNA (rRNA) database by Bowtie2 software and the mapped reads were removed. The remaining

Table 1
Statistics of the reads and comparison to the silkworm reference genome after removing the rRNA reads of each sample.

Sample	Clean data (bp)	High quality clean data (bp)	Q20 (%)	GC (%)
PBS	7,507,508,400	7,452,964,410	7,350,394,416 (98.62%)	3,471,219,215 (46.58%)
TT01	7,696,817,400	7,648,728,163	7,556,903,464 (98.80%)	3,677,305,335 (48.08%)
H06	7,795,408,800	7,742,611,779	7,638,616,870 (98.66%)	3,625,145,695 (46.82%)

Sample	Clean reads num	High quality clean reads num (%)	Adapter (%)	Low quality (%)
PBS	50,050,056	49,846,412 (99.59%)	23,704 (0.1%)	89,678 (0.36%)
TT01	51,312,116	51,162,416 (99.71%)	26,682 (0.1%)	61,239 (0.24%)
H06	51,969,392	51,766,738 (99.61%)	24,166 (0.1%)	88,934 (0.34%)

Sample	Total reads	Unique mapped reads	Multiple mapped reads	Mapping ratio
PBS	49,682,226	43,776,980 (88.11%)	595,396 (1.20%)	89.31%
TT01	50,929,762	44,700,475 (87.77%)	703,486 (1.38%)	89.15%
H06	51,616,142	45,404,743 (87.97%)	641,500 (1.24%)	89.21%

reads were mapped to the reference sequence by TopHat2 software (Kim et al., 2013). Gene abundances were quantified by software RSEM. The gene expression level was normalized by the number of uniquely mapped fragments per kilobase of exon region per million mappable reads (FPKM). Therefore, the FPKM value can be directly used for comparing the differences in gene expression among samples and the formula was defined as below:

$$FPKM = \frac{10^6 C}{NL/10^3}$$

Here, the C, N and L were referred to the number of fragments uniquely mapped to the given gene, the number of reads uniquely mapped to all genes and the total length of exons from the given gene (Langmead and Salzberg, 2012; Li and Dewey, 2011; Xia et al., 2004).

2.5. Screening of different gene expression (DEGs)

DEGs were found out between the immune-primed (TT01 and H06) and the non-primed (PBS) libraries according to a statistical analysis of the frequency of each transcript and their corresponding *P*-values were performed with methods described by Audic and Claverie (1997), The significance threshold of P-value in multiple tests was set by false discovery rate (FDR). We identified genes with a fold change ≥2 and $p < .05$ in a comparison as significant DEGs.

2.6. GO and KEGG enrichment of DEGs

To determine the main biological functions of DEGs, GO enrichment analysis of functional significance terms in the GO database was applied using hypergeometric test to find significantly enriched GO terms in DEGs comparing to the genome background. For the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, we mapped all DEGs to terms in the KEGG database and looked for significantly enriched KEGG terms (Kanehisa et al., 2010).

2.7. Expression validation using qRT-PCR

In order to validate the reliability of RNA-Seq data, qRT-PCR of 25 differentially expressed immune-related genes were performed using a Bio-Rad IQ5 Real-Time PCR system with SYBR-Green detection (SYBR Premix, TIANGEN). Total RNA from control and tested samples used for the qRT-PCR were the same as for RNA-Seq. First cDNA was synthesized and the PCR was performed using the PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Japan). The reaction program consists of two steps: 42 °C for 15 min and then 95 °C for 3 min. The PCR cycling parameters are as follows: initial denaturation at 95 °C for 90 s, followed by

40 cycles of 5 s at 95 °C, 15 s at 60 °C and 20 s at 72 °C. The actin-52-like gene was used to normalize expression levels, and the relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Cheng et al., 2008). All the fluorescence quantitative primers were designed using Primer 5.0 software according to rigorous criteria. The primer information was provided in Table S1.

3. Results

3.1. Sequencing and assembly of illumina short reads

Three cDNA libraries were generated with mRNA from the haemocyte samples: control (PBS), TT01 (primed with heat-killed TT01 cells), H06 (primed with heat-killed H06 cells). These cDNA libraries were then sequenced using an Illumina HiSeq2500 platform. After filtering the raw reads and removing the rRNA mapped reads, approximately 49.7, 50.9, 51.6 million clean reads with a Q20 percentage (those with a base quality > 20) over 98% were generated from PBS, TT01 and H06 cDNA libraries, which encompassed 7.5, 7.7 and 7.8 gigabases (Gb) sequencing data, respectively (Table 1 and Table S2). The data sets supporting the results of this study are available at the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) repository under accession number: SRP108457. Approximately 89% of the sequenced reads of each sample were successfully mapped to the reference genome sequence using TopHat2 software. Of these, approximately 88% were uniquely mapped reads and 1.2% was multiple mapped reads (Table 1). The saturation, sequencing random distribution and distribution of genes' coverage were analyzed to evaluate the quality of each library (Figs. S1–S3).

3.2. DEGs and functional analysis

We identified 338, 59 and 144 up-regulated DEGs and 369, 100 and 317 down-regulated DEGs in PBS-vs-TT01, PBS-vs-H06 and TT01-vs-H06, respectively, with fold changes of > 2 or < 0.5 and $p < .05$ (Fig. 1). To further investigate the biological functions of DEGs, gene ontology (GO) and functional enrichment analysis were performed to map all the DEGs to terms in the GO, euKaryotic Ortholog Groups of proteins (KOG) and Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG) database.

The results of GO enrichment analyses were presented in Fig. 2. The DEGs in PBS-vs-TT01, PBS-vs-H06 and TT01-vs-H06 were assigned to 46, 35 and 42 functional groups respectively, and classified into three GO categories: Biological process, Cellular component, and Molecular function, In the biological process category, 'Cellular process' and 'Metabolic process' were the two most representative subcategories. In
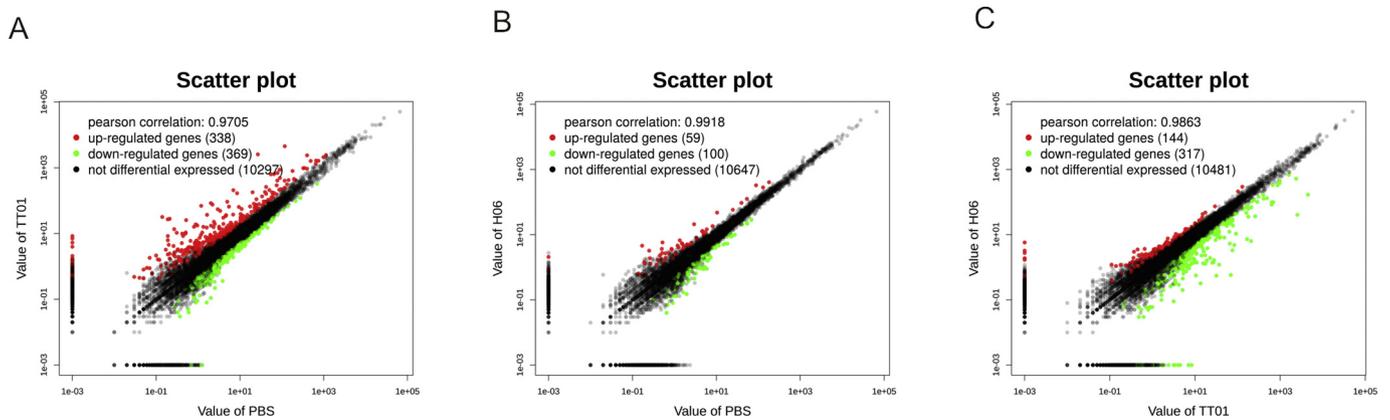
A

Scatter plot

B

Scatter plot

C

Scatter plot

Fig. 1. Scatter plot analysis of three sample pairs (A: PBS-vs-TT01, up-regulated expression of TT01 relative to PBS; B: PBS-vs-H06, up-regulated expression of H06 relative to PBS; and C: TT01-vs-H06, up-regulated expression of H06 relative to TT01) from haemocytes of *Bombyx mori* larvae. The horizontal and vertical co-ordinates represent the expressions of two samples. The red (up-regulated expression) and green (down-regulated expression) points indicate that the gene expression level is different (the judgment standard is $p < .05$, and the difference multiple is more than two times), while the black point shows no difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cellular component category, DEGs related to 'Cell' and 'Cell part' were dominant, while in the molecular function category, the majority of DEGs were involved in 'Catalytic activity' and 'binding'.

The DEGs were aligned to the KOG database to predict and classify possible functions. All the DEGs derived from PBS-vs-TT01, PBS-vs-H06 and TT01-vs-H06 were classified into 25 functional categories. Except for the poorly characterized proteins ('general function prediction only' and 'function unknown'), 'Signal transduction mechanisms' represented the largest group. Following that, the 'Posttranslational modification, protein turnover, chaperones', 'Amino acid transport and metabolism' and 'Intracellular trafficking', secretion, and 'Vesicular transport' were the majority in the PBS-vs-TT01; the 'Posttranslational modification, protein turnover, chaperones', 'Cytoskeleton' and 'Intracellular trafficking, secretion, and vesicular transport' constituted majority parts in the PBS-vs-H06, whereas the 'Amino acid transport and metabolism', 'Posttranslational modification, protein turnover, chaperones' and 'Carbohydrate transport and metabolism' accounted for the major part of functional categories in the TT01-vs-H06 (Fig. 3).

To identify the biological pathways that are involved in the specific immune priming of *B. mori*, KEGG pathways classification was performed. The results showed that 147, 27 and 85 DEGs for PBS-vs-TT01, PBS-vs-H06 and TT01-vs-H06 were enriched in 29, 22 and 19 KEGG pathways respectively. On the basis of the DEGs involved in the KEGG pathways, they were classified into six categories which included Metabolism, Genetic information processing, Environmental information processing, Cellular processes, Organismal systems, Human diseases (Fig. 4). We listed the 6 most significantly enriched pathways (Table 2) of each group and found that a large number of DEGs were enriched in phagosome pathway.

3.3. Putative genes and pathways involved in specific immune priming in silkworm

High-throughput sequencing effort revealed that six main groups of genes involved in pattern recognition receptors (PRRs), AMPs, signaling molecular, effector molecules, phagosome and spliceosome participated in the regulation of immune priming reaction in silkworm. For example, in the PBS-vs-TT01 group, we totally identified at least 12 PRPs transcripts including 5 peptidoglycan recognition proteins (PGRPs), 2 Beta-1, 3-Glucan recognition proteins (βGRPs), 1 cuticular protein tweedle motif 1 precursor (CPT-1), 1 leucine-rich repeat-containing G-protein coupled receptor 5 (LGR-5), 1 hemocytins, 1 Down syndrome cell adhesion molecule-like protein (Dscam 2) and 1 5-hydroxytryptamine receptor 1A (5-HTR). We found that 14 AMPs containing cecropin,

attacin, moricin, gloverin, lebocin and lysozyme were all significantly up-regulated, indicating they play an important role in the immune priming response. A large number of molecules involved in immune signaling pathways such as MyD88, Cactus and KappaB-Ras were also identified in the PBS-vs-TT01 group. Moreover, many genes involved in phagosome, spliceosome and stress responses, including integrin beta2, alpha-tubulin, Prp22, chaoptin, CYP4G25 and heat shock protein (Hsp) were considered to mediate the immune priming, because they showed significant expression changes in PBS-vs-TT01 (Table S3).

In the PBS-vs-H06 group, 4 AMPs were identified and three of them including gloverin, lysozyme and the immune-related protein 2 precursor were significantly up-regulated. Moreover, the analysis of digital expression profile showed that 4 PRPs including 1 hemolin, 1 βGRP and 2 chemosensory proteins (CSPs) were obviously up-regulated after the infection of H06, whereas the other 5 PRPs were down-regulated. Number of genes known to be involved in immune-related signaling pathway, immune effector, phagocytosis and alternative splicing had also marked expression variations after immune priming in this group (Table S4).

We found a considerable number of DEGs related to antibacterial activity (e.g., cecropin, attacin, gloverin and moricin), pathogen recognition (e.g., PGRPs and βGRPs) and many other immune effectors (e.g., heat shock protein, cytochrome P450, serine/threonine-protein kinase SIK2-like and peroxisomal membrane protein PEX13) in the TT01-vs-H06 group. We also identified DEGs involved in alternative splicing and hemocyte phagocytosis (Table S5).

3.4. Phagosome signaling pathway

In the insect immune system, phagocytosis is a major mechanism used to remove pathogens and cell debris. When a microorganism or senescent cell engulfed by a phagocyte via phagocytosis, a phagosome will be formed via the fusion of the phagocyte membrane around the particle. In the PBS-vs-TT01 group, integrin beta2, transport protein Sec61 beta subunit, vacuolar H^{+} ATPase subunit 100–1, alpha-tubulin, vesicle-trafficking protein SEC22b-B, GM11995, 1-phosphatidy-linositol 3-phosphate 5-kinase-like, vacuolar ATP synthase subunit G, transport protein Sec61 gamma subunit, integrin beta-6-like and vacuolar ATP synthase 21 kDa proteolipid subunit were the DEGs enriched in phagosome signaling pathway, among which integrin beta2 and integrin beta-6-like were down-regulated (Fig. 5).

In the PBS-vs-H06 group, four DEGs, including ras-related C3 botulinum toxin substrate 1, V-type proton ATPase 116 kDa subunit a isoform 1-like, transport protein Sec61 gamma subunit and integrin
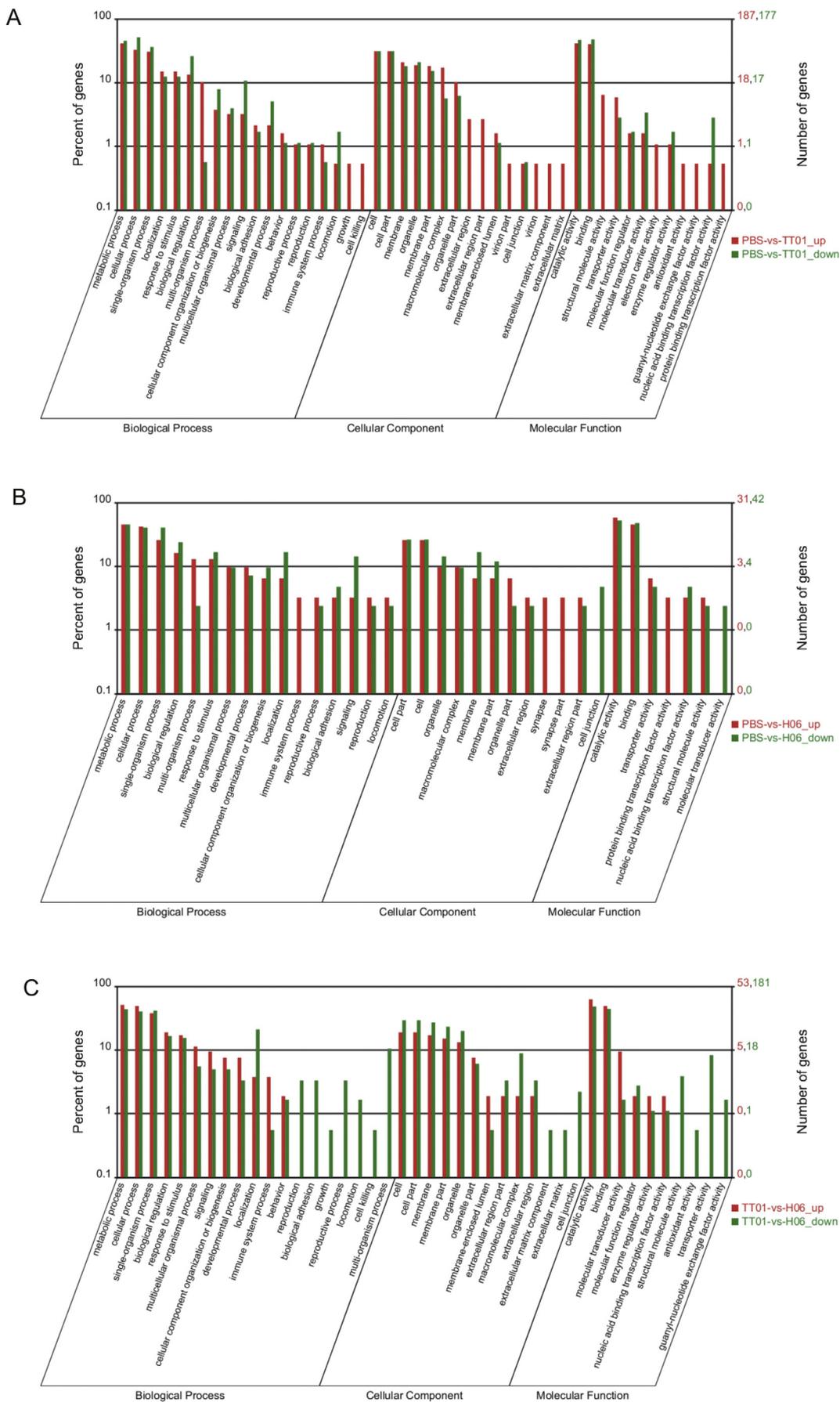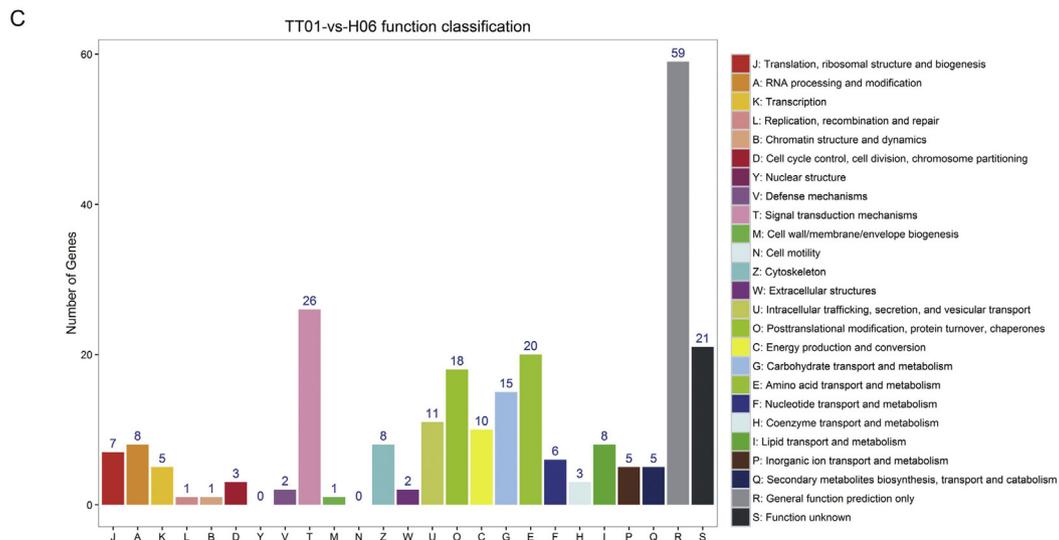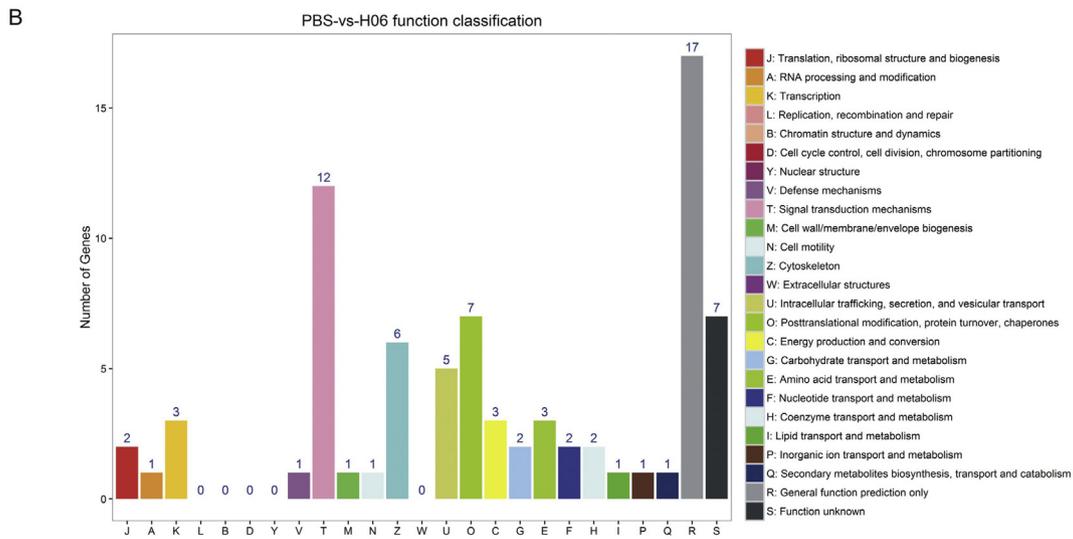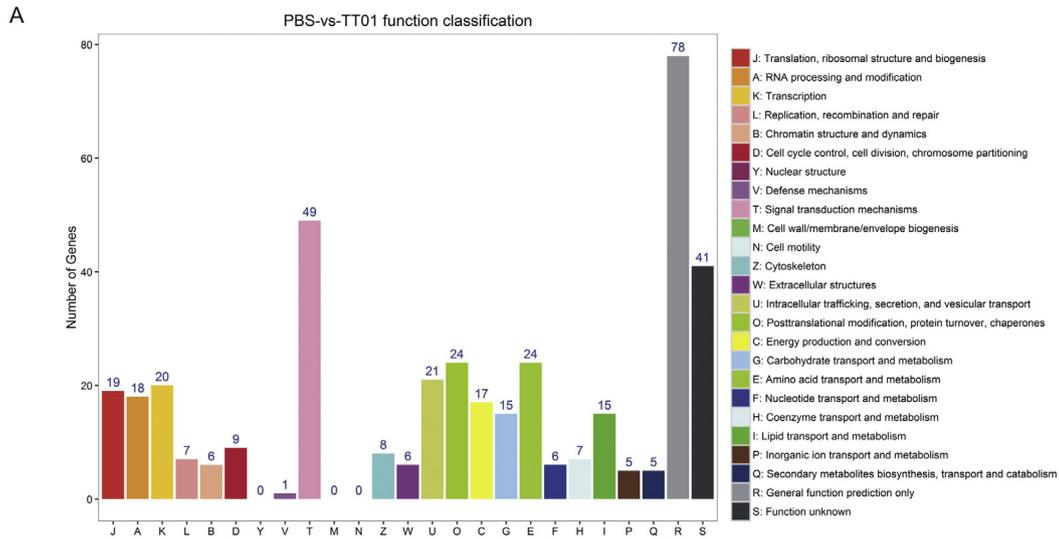
Fig. 2. Gene Ontology (GO) annotations of different expression genes (DEGs) by Blast2GO. The x-axis indicates the subcategories, the left y-axis represents the percentage of a specific category of DEGs and the right y-axis indicates the number of DEGs (A, PBS-vs-TT01; B, PBS-vs-H06 and C, TT01-vs-H06).

(caption on next page)

Fig. 3. Histogram presentation of clusters of euKaryotic Ortholog Groups (KOG) classification. The Y-axis indicates the percentage of unigenes in each cluster. The capital letters in x-axis indicates the KOG categories as listed on the right of the histogram and the y-axis indicates the number of differentially expressed genes (DEGs) (A, PBS-vs-TT01; B, PBS-vs-H06 and C, TT01-vs-H06).

beta-6-like were enriched in phagosome signaling pathway, among which only the integrin beta-6-like was down-regulated (Fig. 6).

6 DEGs involved in phagosome pathway were also identified from TT01-vs-H06, including one up-regulated gene (syntaxin-7) and five down-regulated genes (vacuolar H^{+} ATPase subunit, alpha-tubulin, ras-related C3 botulinum toxin substrate 1, GM11995 and V-type proton ATPase subunit e 2-like) (Table 2 and Table S5)

3.5. qRT-PCR validation of transcriptome analysis

To validate our data of the deep sequencing, 13 DEGs (11 up-regulated and 2 down-regulated) from PBS-vs-TT01 and 12 DEGs (9 up-regulated and 3 down-regulated) from PBS-vs-H06 were selected for quantitative RT-PCR analysis using the same RNA samples as for deep sequencing, the unigenes selected encoded attacin (Bm_nscaf2556_13), gloverin (Bm_nscaf3098_42 and Bm_nscaf3097_74), lebocin (Bm_nscaf2855_231), cytochrome P450 (XLOC_000015), integrin beta2 (Bm_nscaf2847_173), beta-1,3-glucan recognition protein (Bm_nscaf3031_280 and Bm_nscaf3031_283), peptidoglycan recognition protein (Bm_nscaf3058_127), cactus (Bm_nscaf2839_39), cecropin (Bm_nscaf1071_24), hemocytin (XLOC_001602), cuticular protein tweedle motif (Bm_nscaf1690_159), alpha-tubulin (Bm_nscaf2511_187), MyD88 (XLOC_006311), immune-related Hdd1 (Bm_nscaf3099_107), lysozyme precursor (Bm_nscaf2993_097), tyrosine hydroxylase (Bm_nscaf1690_113), transport protein Sec61 gamma subunit (Bm_nscaf3093_09), geminin-like (Bm_nscaf1690_121), wing disc-specific protein (Bm_nscaf2674_266), heat shock protein 68 (Bm_scaffold779_2), cytochrome P450 CYP4G25 (Bm_nscaf1898_198), hemolin (Bm_nscaf2916_67) and endoplasmic reticulum oxidoreduction 1-like precursor (Bm_nscaf2575_128). The qPCR results confirmed the data obtained from deep sequencing analysis and showed similar trends in up- or down-regulated unigenes (Fig. 7).

4. Discussion

In our previous study, we have demonstrated that the *B. mori* larvae possessed an immune priming response and revealed a degree of specificity to the TT01 and H06 bacteria, and we found that both the cellular and the humoral immunity were important contributors to the immune priming, whereas the specificity of the priming response was primarily depended on haemocyte phagocytosis (Wu et al., 2015b). In the present study, to gain insights into the mechanism of specific immune priming in *B. mori* larvae at the molecular level, we investigated the whole transcriptome of the immune-primed and non-primed silkworm by Illumina/Solexa next generation sequencing. Using the same strategies, Zhao et al. constructed the transcriptome of the haemocytes and fat body of *Helicoverpa armigera* larvae to identify the DEGs after immune priming (Zhao et al., 2013). However, the potential genes mediated the specificity of the priming and the regulatory pathways had not been revealed, because of no genomic information for *H. armigera* and only one elicitors was used in the study.

There are two main scenarios for the formation of immune priming responses in invertebrates (Milutinovic et al., 2016). In the first scenario, after immunization, the host body forms a large number of immune molecules and maintains at a high level until the secondary challenge occurs. In this case, the survival benefit upon secondary exposure is a consequence of such previously activated defences. Indeed, we found support for this scenario in our dataset. For example, several genes involved in pathogen recognition, antimicrobial activity, immune signal transduction, phagosome and spliceosome were found activated after priming with TT01 and H06, and exhibited distinct expression patterns in different treatments. Such a scenario was recently observed in oral immune priming in red flour beetle and also trans-generational immune priming in bumble bees (Barribeau et al., 2016; Greenwood et al., 2017).

The first step in activating the insect immune system is the PAMPs are effectively recognized by PRRs. In this study, a total of 12 PRPs gene transcripts, which are involved in the immune signaling pathway, are found to be upregulated in the TT01 prming group, including the PGRPs (Bm_nscaf3058_127, Bm_nscaf1690_061, Bm_nscaf2889_001, Bm_nscaf2889_112, XLOC_015318), βGRPs (Bm_nscaf1681_013, Bm_nscaf3031_280), CPT-1 (Bm_nscaf1690_159), LGR-5 (Bm_nscaf2589_017), hemocytins (XLOC_001602), dscam (Bm_nscaf2838_092) and 5-HTR (Bm_nscaf3032_065). However, we only observed upregulations in the expression of four PRRs including 1 βGRPs (Bm_nscaf1681_013), 2 CSP (Bm_nscaf2767_026, Bm_nscaf2767_055) and 1 hemolin (Bm_nscaf2916_67) in the H06 priming group. Previous study on silkworms infected by *Nosema*
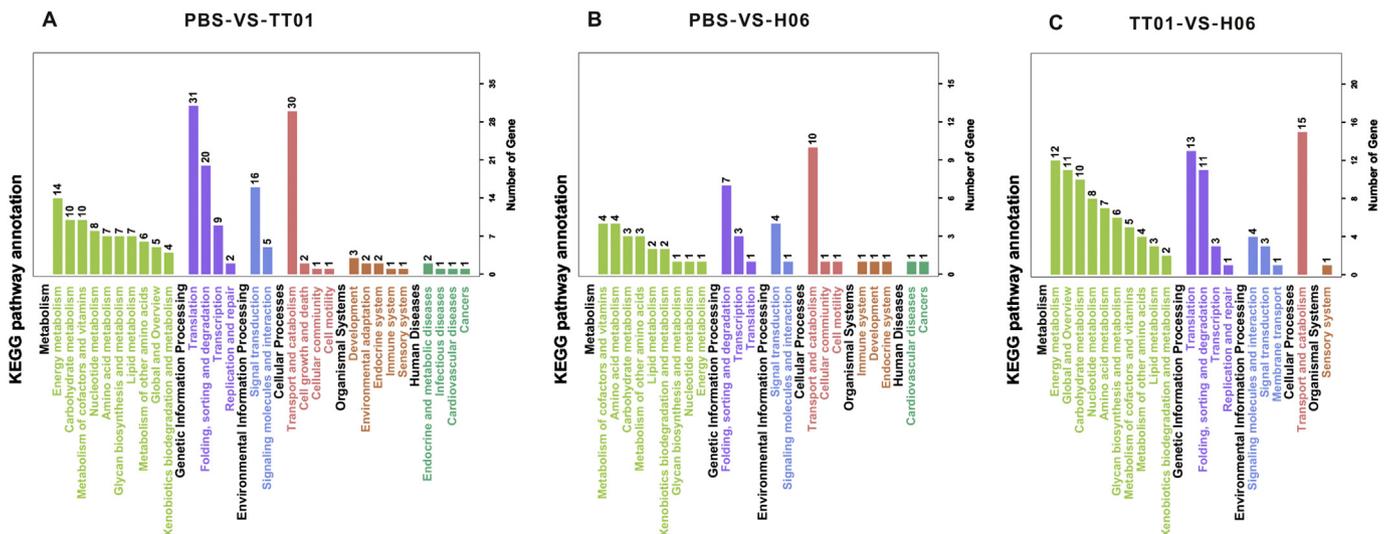

Fig. 4. KEGG Orthology (KO) classifications of differentially expressed genes (DEGs). (A, PBS-vs-TT01; B, PBS-vs-H06 and C, TT01-vs-H06).

Table 2
The 6 most significantly enriched pathways of differentially expressed genes of *Bombyx mori* haemocytes after priming with TT01 and H06.

Sample	Pathway	DEGs genes with pathway annotation	All genes with pathway annotation	P value	Pathway ID
PBS-vs-TT01	Phagosome	11 (7.48%)	88 (2.86%)	0.002741	ko04145
	Oxidative phosphorylation	12 (8.16%)	122 (3.97%)	0.012511	ko00190
	Sulfur relay system	3 (2.04%)	11 (0.36%)	0.013341	ko04122
	Protein export	4 (2.72%)	21 (0.68%)	0.015934	ko03060
	RNA transport	12 (8.16%)	136 (4.43%)	0.027463	ko03013
	mRNA surveillance pathway	8 (5.44%)	80 (2.6%)	0.035506	ko03015
PBS-vs-H06	Endocytosis	5 (18.52%)	120 (3.91%)	0.003385	ko04144
	Phagosome	4 (14.81%)	88 (2.86%)	0.006666	ko04145
	Protein processing in endoplasmic reticulum	5 (18.52%)	153 (4.98%)	0.00951	ko04141
	Ascorbate and aldarate metabolism	2 (7.41%)	28 (0.91%)	0.024435	ko00053
	Osteoclast differentiation	1 (3.7%)	3 (0.1%)	0.026145	ko04380
	Drug metabolism - cytochrome P450	2 (7.41%)	33 (1.07%)	0.033226	ko00982
TT01-vs-H06	Oxidative phosphorylation	11 (12.94%)	122 (3.97%)	0.000433	ko00190
	Phagosome	6 (7.06%)	88 (2.86%)	0.033333	ko04145
	Biosynthesis of amino acids	6 (7.06%)	88 (2.86%)	0.033333	ko01230
	Sulfur relay system	3 (3.53%)	11 (0.36%)	0.002873	ko04122
	Folate biosynthesis	3 (3.53%)	21 (0.68%)	0.018972	ko00790
	Glycosaminoglycan biosynthesis - chondroitin sulfate/dermatan sulfate	2 (2.35%)	11 (0.36%)	0.035389	ko00532

bombycis also demonstrated that many PRPs including βGRP2, βGRP4, PGRP-S3 and PGRP-S4 are significantly activated after infection (Xiong et al., 2015). The increased expression of PRPs showed that the silkworm recognized the entered bacteria and then transmitted the signal to the insect immune system. Therefore, the different expression profiles of the PRRs genes in the two priming groups indicated that the silkworm has different recognition mechanisms for bacteria TT01 and H06. The up-regulated PRRs enable the immune primed insect a quicker and stronger immune response upon a secondary infection of pathogens. So we believed that the PRRs must be involved in the
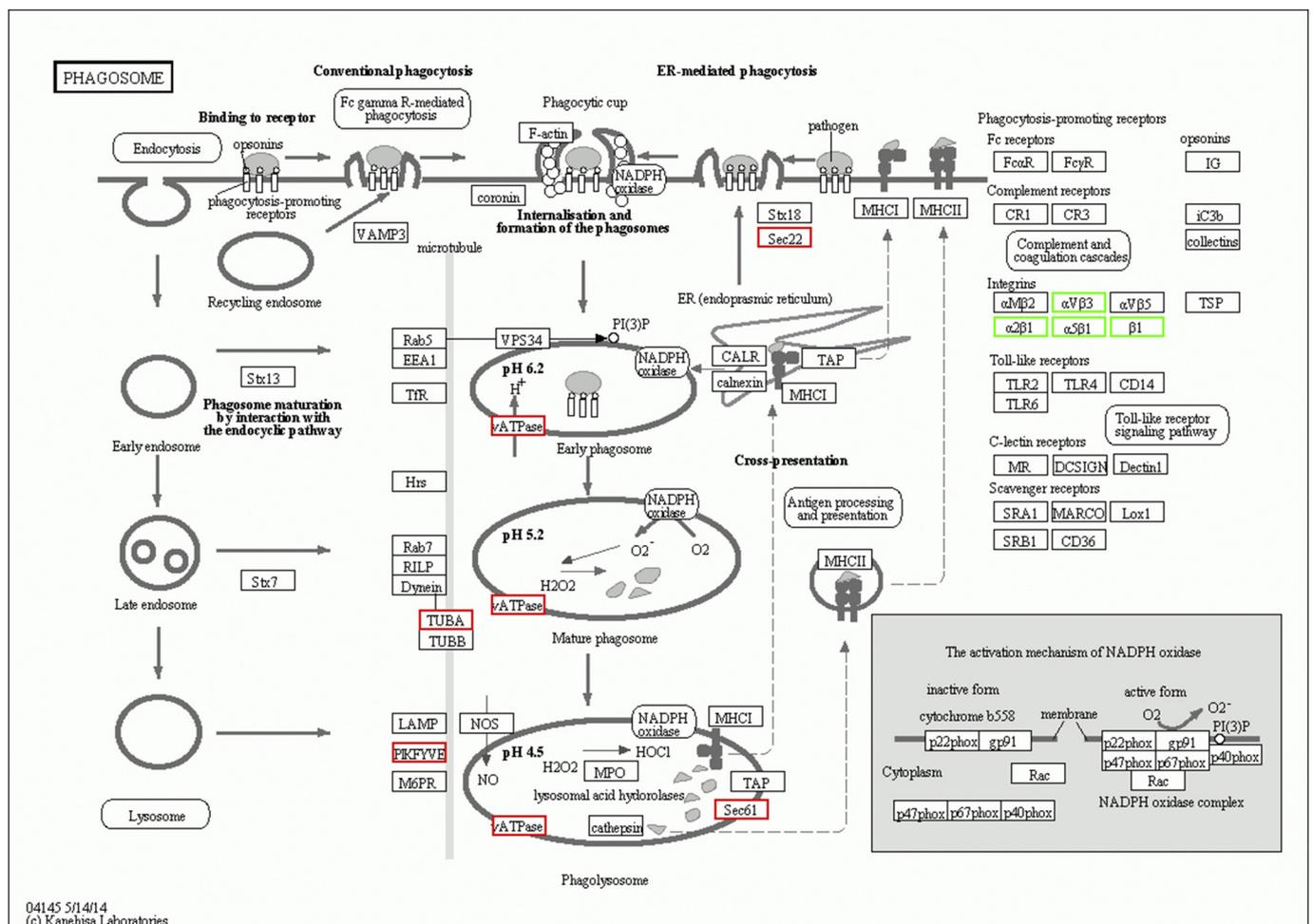

Fig. 5. Differentially expressed genes (DEGs) of PBS-vs-TT01 enriched in the phagocytosis signaling pathways. Red boxes represent up-regulated genes, and green boxes represent down-regulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Differentially expressed genes (DEGs) of PBS-vs-H06 enriched in the phagocytosis signaling pathways. Red boxes represent up-regulated genes, and green boxes represent down-regulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
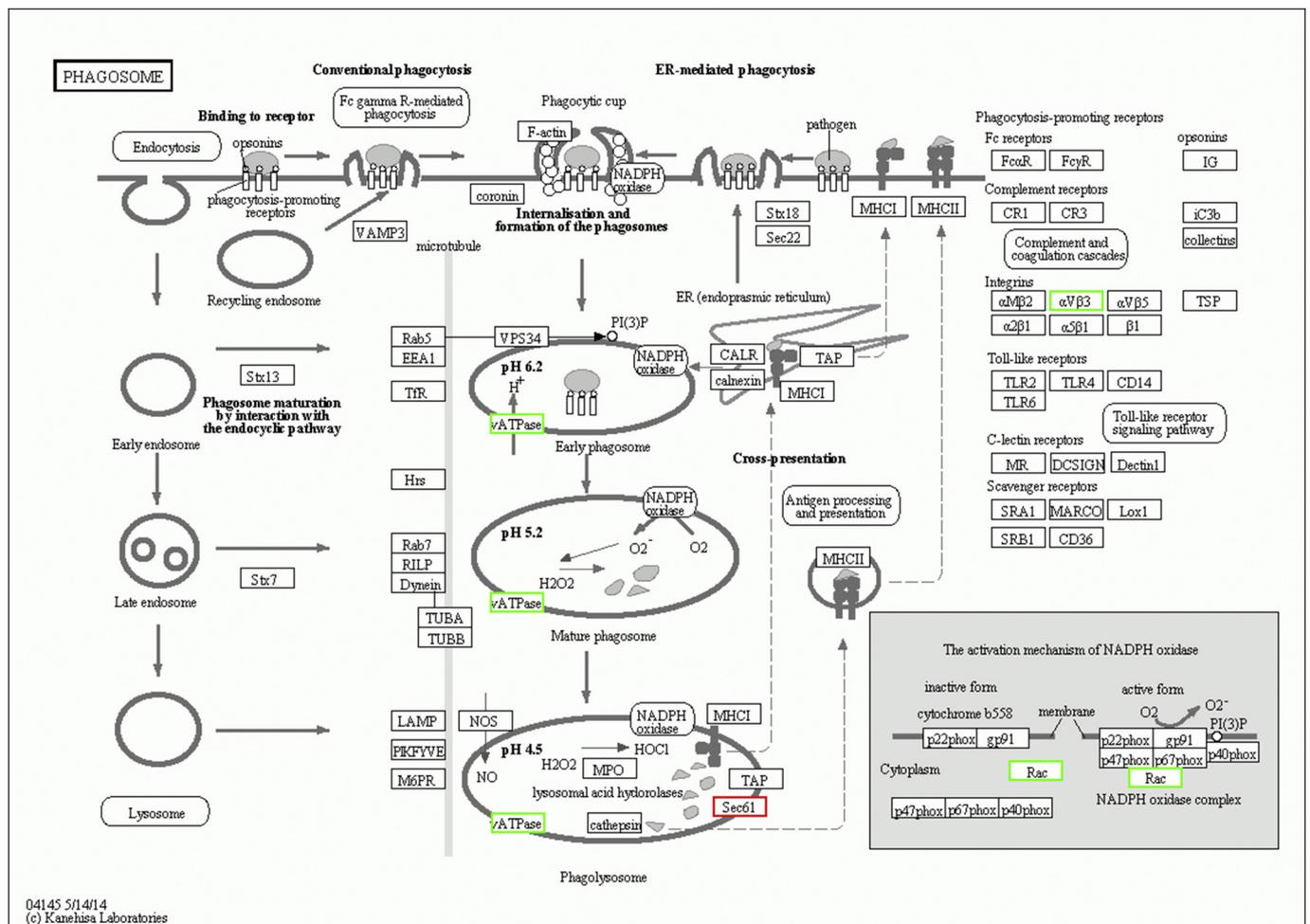
regulation of immune priming. Moreover, previous study also demonstrated that the receptor repertoire can mediate the distinction of different types of pathogens (Cherry and Silverman, 2006). For example, PGRPs can bind to peptidoglycan, a component of the Gram-positive bacteria envelope (Bischoff et al., 2004; Michel et al., 2001), βGRPs bind with high affinity to the Gram-negative bacterium and fungal pathogen (Jiang et al., 2004; Wang et al., 2006; Zheng and Xia, 2012). Although the total number of putative recognition receptors of *B. mori* larvae is very limited, they can efficiently interact with each other than act separately (Schulenburg et al., 2007). These synergistic effects permit the immune system generate a higher degree of specificity. So the diffferent expressing parttern of PRRs in TT01 and H06 prming groups indicated they also contribute to the regulation of specificity of the immune priming.

Haemocytes participate in phagocytosis and contain several types of granules. The granules contain abundant antimicrobial peptides. In the process of phagocytosis, the granules are secreted into the haemolymph where their contents kill microbes or inhibit their multiplication (Iwanaga et al., 1994). In silkworms, six families of AMPs (defensin, attacin, gloverin, cecropin, moricin, and lebocin) have been identified and were found to be upregulated in response to both gram-positive and gram-negative bacteria (Huang et al., 2009; Kaneko et al., 2007; Tanaka et al., 2008). In this study, we found genes encoding AMPs such as cecropin, attacin, gloverin, moricin and lysozyme were 1.5- to 4.5-fold up-regulated in TT01 compared to PBS group. Only two AMPs (gloverin 3 and lysozyme) were significantly up-regulated in the H06 prming group. And also significant down-regulation of cecropin, gloverin,

attacin and moricin were identified in the TT01-vs-H06 group. This is in line with our previous observation that the cell-free haemolymph of TT01 and H06 immune-primed *B. mori* larvae showed a significant higher antimicrobial activity than that of PBS control (Wu et al., 2015b), and is also in accordance with our another study, which reported that immune priming was achieved mainly by the regulation on the quantity and activity of immune parameters including antimicrobial peptides (Wu et al., 2014). High levels of AMPs in the haemocytes enhance the protection of immune-primed larvae against the subsequent infection of the pathogen. There is also increasing evidence that antimicrobial peptides released in response to microbial invasion can activate adaptive immunity in higher vertebrates (Ganz, 2003). For these reasons, the enhanced immune priming elicited by TT01 and H06 is also attributed to the differential expression of the antimicrobial peptide genes in *B. mori* larvae haemocytes. Although it is still unclear to what extent such antimicrobial activity could confer highly specific immune protection, we believe that the combination of AMPs could potentially mediate higher specificity than single AMPs. Because synergistic interactions of the immune effectors can generate a specific immune priming (Schulenburg et al., 2007). And the different expression of a large number of antimicrobial peptide genes in TT01-vs-H06 group supports this conclusion.

Many studies reported that phagocytic ability of haemocytes significantly increased against the reinfection of pathogens and the increased phagocytic responses have features of specificity to recognize the same pathogen in the immune priming of *Drosophila* (Pham et al., 2007), shrimp (Pope et al., 2011), woodlouse (Roth and Kurtz, 2009)
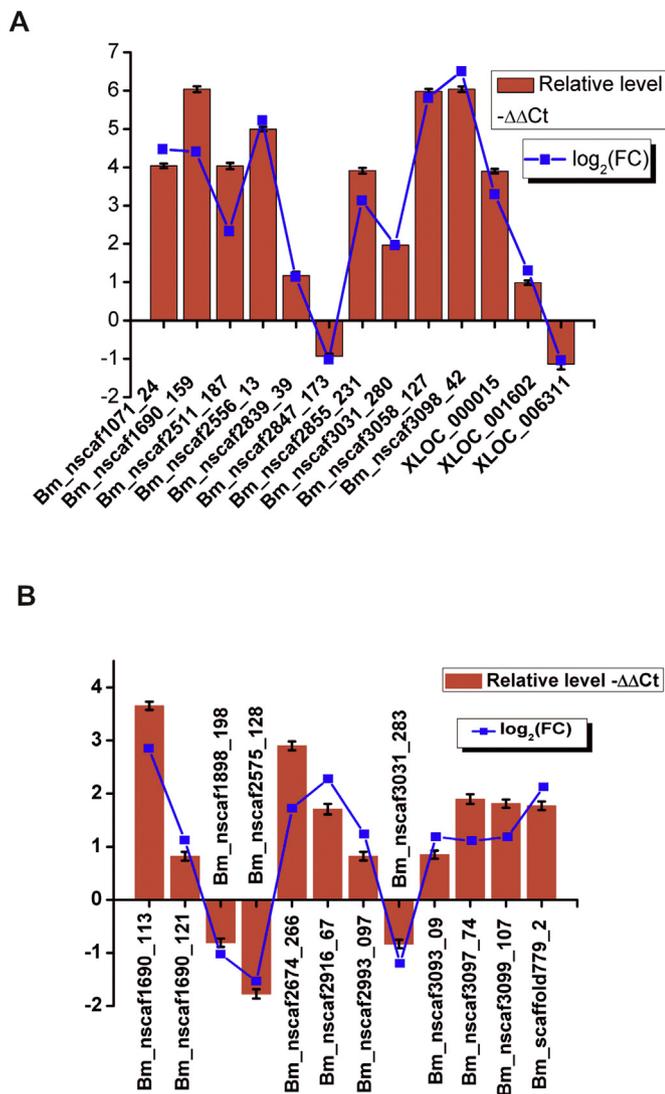
A

B

Fig. 7. qPCR validation of the selected DEGs. The y-axis indicates the value of relative expression level ($2^{-\Delta\Delta Ct}$) by qPCR and $\log_2$Ratio of TT01/PBS (A) or H06/PBS (B) by deep sequencing.

and silkworm (Wu et al., 2015b). The sequencing data in present study provided molecular evidence that phagocytosis plays an important role in the regulation of specific immune priming. Through comparison of two priming groups and PBS group, we found 20 (12 up-regulated and 8 down-regulated) and 9 (5 up-regulated and 4 down-regulated) DEGs in the TT01 and H06 priming group respectively involved in phagosome or endocytosis signaling pathways. Among the DEGs mediated the phagocytosis and endocytosis, only three DEGs (Bm_nscaf3093_09, Bm_scaffold731_1 and Bm_nscaf2888_375) can be found and showed similar expression levels in the two immune-priming groups, the DEGs related to phagocytosis and endocytosis detected in this study supported the view that the specificity of immune priming in silkworm was primarily attributed to the haemocyte phagocytosis (Wu et al., 2015b).

A large number of signaling molecules associated with immune defence including immune-related Hdd1 were found strongly expressed upon TT01 or H06 priming, this is also consistent with a previous study which found that HDD1 was dramatically induced in the haemocytes of *B. mori* larvae after injection with bacteria or many other PAMPs (Zhang et al., 2017). Interestingly, a gene of the same class (Hdd23) was found induced in the TT01 priming group, but not detected in the H06 priming group, indicating its importance in regulating the specificity of immune priming. Compared to control group, the expression levels of

genes involved in Toll signaling pathway, such as cactus and MyD88 were changed dramatically after immune priming with TT01, whereas no significant differences was detected in the H06 priming group. This difference can be caused by the different components on the cell surface of TT01 and H06 (Duchaud et al., 2003). Pham et al. reported that the specific immune priming in *D. melanogaster* was only observed against certain bacterial species and mediated by phagocytosis and Toll pathway (Pham et al., 2007).

We identified a number of genes, which functions as immune effectors were up-regulated during the immune-priming process. For example, the tyrosine hydroxylase (TH) were 4.2- and 2.8-fold up-regulated in TT01 and H06 prming group respectively, This result suggests that TH is required for the *B. mori* larvae immune priming responses because TH is a major producer of the 3,4-dihydroxyphenylalanine required for immune-associated melanization (Gorman et al., 2007). We also found that a considerable number of immune effectors associated with stress adaptation or detoxification were up- or down-regulated after the larvae priming with TT01 or H06, including heat shock protein, cytochrome P450 CYP4G25, glucose dehydrogenase, serine protease (De Gregorio et al., 2001; Zhang et al., 2012). The transcriptome data in this study provided evidence that the immune effectors play an important function in mediating the priming response and also confirmed previous finding that the interdependencies between immune and stress responses exist in insect (Altincicek et al., 2013; Altincicek and Vilcinskas, 2007).

Although insects lack somatic recombination mechanisms, it has been demonstrated that several immune receptors such as fibrinogen-related proteins (FREPS) and DSCAM can generate diversity through alternative splicing (Cerenius and Soderhall, 2013; Zhang et al., 2004). The dramatically different expression levels of genes involved in spliceosome identified in this study provided further confirmation for these conclusions. Furthermore, we detected that the expression level of *Dscam* gene was significantly up-regulated in the TT01 priming group compared the PBS control, whereas no significant difference was observed in the H06 priming group. The reason for the different results observed in two immune priming groups was mainly attributed to the various PAMPs on the cell wall of the bacteria TT01 and H06. Indeed, studies on *A. gambiae* have shown that Dscam was specific to some degree in terms of binding to the bacteria (Dong et al., 2006), another study using bumblebee *Bombus terrestris/*trypanosome *Crithidia bombi* as a model demonstrated that the expression and alternative splicing of Dscam is associated with specific interactions between different host and parasite genotypes (Riddell et al., 2014). These, combined with our results, demonstrated that Dscam play an important role in the immune priming response. Further research is needed to see how this interesting gene is involved in regulation of immune priming in *B. mori* larvae.

Although the DEGs obtained in present study revealed critical components in the signal pathway and provided valuable clues for investigating the regulatory pathways of immune priming, it is not possible to reveal the networks of these pathways based on the data obtained now because of the following reasons: The whole cell of TT01 and H06 that contained many pathogen-associated molecular patterns (PAMPs) (such as lipopolysaccharide and peptidoglycan) was injected into the insect as antigen that would trigger many signaling pathways thus increased the complexity of the study. In our future study, we will extract and purify the PAMPs from TT01 and H06 to reveal the common pathways or networks involved in the priming responses.

In summary, we here show that immune priming responses for TT01 and H06 is achieved by extensive transcriptome changes in *B. mori* larvae haemocyte. Large number genes involved in antimicrobial activity, pathogen recognition, immune effector, immune signal transduction, phagosome and spliceosome were identifed based on bioinformatics analysis and found to play important role in mediating the specific immune priming of *B. mori* larvae. Although the in vivo functions of these genes still need to be validated by molecular studies, such as gain-of-function or loss-of-function analysis. We have generated

information about the genetic composition and regulation of the immune priming and specificity in insects. Exploring the molecular mechanism of specific immune priming of invertebrates will help us control insect pests or prevent epidemic of infectious diseases in economic invertebrates in the future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103921.

Declaration of Competing Interest

None.

References

Altincicek, B., Vilcinskas, A., 2007. Analysis of the immune-inducible transcriptome from microbial stress resistant, rat-tailed maggots of the drone fly Eristalis tenax. BMC Genomics 8.

Altincicek, B., Elashry, A., Guz, N., Grundler, F.M., Vilcinskas, A., Dehne, H.W., 2013. Next generation sequencing based transcriptome analysis of septic-injury responsive genes in the beetle Tribolium castaneum. PLoS One 8, e52004.

Audic, S., Claverie, J.M., 1997. The significance of digital gene expression profiles. Genome Res. 7, 986–995.

Bai, X., Mamidala, P., Rajarapu, S.P., Jones, S.C., Mittapalli, O., 2011. Transcriptomics of the bed bug (Cimex lectularius). PLoS One 6, e16336.

Bang, K., Hwang, S., Lee, J., Cho, S., 2015. Identification of immunity-related genes in the larvae of Protaetia brevitarsis seulensis (Coleoptera: Cetoniidae) by a next-generation sequencing-based transcriptome analysis. J. Insect Sci. 15.

Bao, Y.Y., Wang, Y., Wu, W.J., Zhao, D., Xue, J., Zhang, B.Q., Shen, Z.C., Zhang, C.X., 2012. De novo intestine-specific transcriptome of the brown planthopper Nilaparvata lugens revealed potential functions in digestion, detoxification and immune response. Genomics 99, 256–264.

Barribeau, S.M., Schmid-Hempel, P., Sadd, B.M., 2016. Royal Decree: gene expression in trans-generationally immune primed bumblebee workers mimics a primary immune response. PLoS One 11, e0159635.

Bischoff, V., Vignal, C., Boneca, I.G., Michel, T., Hoffmann, J.A., Royet, J., 2004. Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria. Nat. Immunol. 5, 1175–1180.

Boraschi, D., Italiani, P., 2018a. Innate immune memory: time foe adopting a correct terminology. Front. Immunol. 9.

Boraschi, D., Italiani, P., 2018b. Innate immune memory: time for adopting a correct terminology. Front. Immunol. 9, 799.

Cerenius, L., Soderhall, K., 2013. Variable immune molecules in invertebrates. J. Exp. Biol. 216, 4313–4319.

Cheng, T.C., Zhang, Y.L., Liu, C., Xu, P.Z., Gao, Z.H., Xia, Q.Y., Xiang, Z.H., 2008. Identification and analysis of toll-related genes in the domesticated silkworm, Bombyx mori. Dev. Comp. Immunol. 32, 464–475.

Cherry, S., Silverman, N., 2006. Host-pathogen interactions in drosophila: new tricks from an old friend. Nat. Immunol. 7, 911–917.

Cooper, D., Eleftherianos, I., 2017. Memory and specificity in the insect immune system: current perspectives and future challenges. Front. Immunol. 8.

De Gregorio, E., Spellman, P.T., Rubin, G.M., Lemaitre, B., 2001. Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc. Natl. Acad. Sci. U. S. A. 98, 12590–12595.

Dong, Y.M., Taylor, H.E., Dimopoulos, G., 2006. AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system. PLoS Biol. 4, 1137–1146.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Medigue, C., Lanois, A., Powell, K., Siguier, P., Vincent, R., Wingate, V., Zouine, M., Glaser, P., Boemare, N., Danchin, A., Kunst, F., 2003. The genome sequence of the entomopathogenic bacterium Photorhabdus luminescens. Nat. Biotechnol. 21, 1307–1313.

Eleftherianos, I., Marokhazi, J., Millichap, P.J., Hodgkinson, A.J., Sriboonlert, A., ffrench-Constant, R.H., Reynolds, S.E., 2006. Prior infection of Manduca sexta with non-pathogenic Escherichia coli elicits immunity to pathogenic Photorhabdus luminescens: roles of immune-related proteins shown by RNA interference. Insect Biochem. Mol. Biol. 36, 517–525.

Etebari, K., Palfreyman, R.W., Schlipalius, D., Nielsen, L.K., Glatz, R.V., Asgari, S., 2011. Deep sequencing-based transcriptome analysis of Plutella xylostella larvae parasitized by Diadegma semiclausum. BMC Genomics 12, 446.

Faulhaber, L.M., Karp, R.D., 1992. A diphasic immune response against bacteria in the American cockroach. Immunology 75, 378–381.

Galvez, D., Chapuisat, M., 2014. Immune priming and pathogen resistance in ant queens. Ecol. Evol. 4, 1761–1767.

Ganz, T., 2003. The role of antimicrobial peptides in innate immunity. Integr. Comp. Biol. 43, 300–304.

Gorman, M.J., An, C.J., Kanost, M.R., 2007. Characterization of tyrosine hydroxylase from Manduca sexta. Insect Biochem. Mol. Biol. 37, 1327–1337.

Greenwood, J.M., Milutinovic, B., Peuss, R., Behrens, S., Esser, D., Rosenstiel, P., Schulenburg, H., Kurtz, J., 2017. Oral immune priming with Bacillus thuringiensis induces a shift in the gene expression of Tribolium castaneum larvae. BMC Genomics 18, 329.

Hernandez Lopez, J., Schuehly, W., Crailsheim, K., Riessberger-Galle, U., 2014. Transgenerational immune priming in honeybees. Proc. Biol. Sci. 281, 20140454.

Hua, X.T., Ma, X.J., Xue, R.J., Cheng, T.C., Wang, F., Xia, Q.Y., 2016. Characterization of the Bombyx mori Cecropin A1 promoter regulated by IMD pathway. J. Insect Sci. 23, 297–304.

Huang, L.L., Cheng, T.C., Xu, P.Z., Cheng, D.J., Fang, T., Xia, Q.Y., 2009. A genome-wide survey for host response of silkworm, Bombyx mori during pathogen Bacillus bombyseptieus infection. PLoS One 4.

Iwanaga, S., Muta, T., Shigenaga, T., Miura, Y., Seki, N., Saito, T., Kawabata, S., 1994. Role of hemocyte-derived granular components in invertebrate defense. Ann. N. Y. Acad. Sci. 712, 102–116.

Jiang, H.B., Ma, C.C., Lu, Z.Q., Kanost, M.R., 2004. B-1,3-Glucan recognition protein-2 (beta GRP-2) from Manduca sexta: an acute-phase protein that binds beta-1,3-glucan and lipoteichoic acid to aggregate fungi and bacteria and stimulate prophenoloxidase activation. Insect Biochem. Mol. Biol. 34, 89–100.

Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M., Hirakawa, M., 2010. KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res. 38, D355–D360.

Kaneko, Y., Furukawa, S., Tanaka, H., Yamakawa, M., 2007. Expression of antimicrobial peptide genes encoding enbocin and gloverin isoforms in the silkworm, Bombyx mori. Biosci. Biotechnol. Biochem. 71, 2233–2241.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Kurtz, J., Armitage, S.A., 2006. Alternative adaptive immunity in invertebrates. Trends Immunol. 27, 493–496.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.

Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinforma. 12, 323.

Li, J., Ma, L., Lin, Z., Zou, Z., Lu, Z., 2016. Serpin-5 regulates prophenoloxidase activation and antimicrobial peptide pathways in the silkworm, Bombyx mori. Insect Biochem. Mol. Biol. 73, 27–37.

Michel, T., Reichhart, J.M., Hoffmann, J.A., Royet, J., 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414, 756–759.

Milutinovic, B., Peuss, R., Ferro, K., Kurtz, J., 2016. Immune priming in arthropods: an update focusing on the red flour beetle. Zoology (Jena) 119, 254–261.

Moreau, J., Martinaud, G., Troussard, J.P., Zanchi, C., Moret, Y., 2012. Trans-generational immune priming is constrained by the maternal immune response in an insect. Oikos 121, 1828–1832.

Moret, Y., Siva-Jothy, M.T., 2003. Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, Tenebrio molitor. Proc. Biol. Sci. 270, 2475–2480.

Morozova, O., Marra, M.A., 2008. Applications of next-generation sequencing technologies in functional genomics. Genomics 92, 255–264.

Netea, M.G., Quintin, J., van der Meer, J.W., 2011. Trained immunity: a memory for innate host defense. Cell Host Microbe 9, 355–361.

Pham, L.N., Dionne, M.S., Shirasu-Hiza, M., Schneider, D.S., 2007. A specific primed immune response in Drosophila is dependent on phagocytes. PLoS Pathog. 3.

Pope, E.C., Powell, A., Roberts, E.C., Shields, R.J., Wardle, R., Rowley, A.F., 2011. Enhanced cellular immunity in shrimp (Litopenaeus vannamei) after 'vaccination. PLoS One 6, e20960.

Reber, A., Chapuisat, M., 2012. No evidence for immune priming in ants exposed to a fungal pathogen. PLoS One 7.

Riddell, C.E., Lobaton Garces, J.D., Adams, S., Barribeau, S.M., Twell, D., Mallon, E.B., 2014. Differential gene expression and alternative splicing in insect immune specificity. BMC Genomics 15, 1031.

Rodrigues, J., 2010. Hemocyte differentiation mediates innate immune memory in Anopheles gambiae mosquitoes. Science 329, 1353 (330, 448–448).

Roth, O., Kurtz, J., 2009. Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse Porcellio scaber (Crustacea: Isopoda). Dev. Comp. Immunol. 33, 1151–1155.

Roth, O., Sadd, B.M., Schmid-Hempel, P., Kurtz, J., 2009. Strain-specific priming of resistance in the red flour beetle, Tribolium castaneum. Proc. R. Soc. B 276, 145–151.

Sadd, B.M., Schmid-Hempel, P., 2006. Insect immunity shows specificity in protection upon secondary pathogen exposure. Curr. Biol. 16, 1206–1210.

Schulenburg, H., Boehnisch, C., Michiels, N.K., 2007. How do invertebrates generate a highly specific innate immune response? Mol. Immunol. 44, 3338–3344.

Tanaka, H., Ishibashi, J., Fujita, K., Nakajima, Y., Sagisaka, A., Tomimoto, K., Suzuki, N., Yoshiyama, M., Kaneko, Y., Iwasaki, T., Sunagawa, T., Yamaji, K., Asaoka, A., Mita, K., Yamakawa, M., 2008. A genome-wide analysis of genes and gene families

involved in innate immunity of Bombyx mori. Insect Biochem. Mol. Biol. 38, 1087–1110.

Taniai, K., Furukawa, S., Shono, T., Yamakawa, M., 1996. Elicitors triggering the simultaneous gene expression of antibacterial proteins of the silkworm, Bombyx mori. Biochem. Biophys. Res. Commun. 226, 783–790.

Tidbury, H.J., Pedersen, A.B., Boots, M., 2011. Within and transgenerational immune priming in an insect to a DNA virus. Proc. R. Soc. B 278, 871–876.

Vogel, H., Badapanda, C., Knorr, E., Vilcinskas, A., 2014. RNA-sequencing analysis reveals abundant developmental stage-specific and immunity-related genes in the pollen beetle Meligethes aeneus. Insect Mol. Biol. 23, 98–112.

Wago, H., 1983. Cellular recognition of foreign materials by Bombyx mori phagocytes: II. Role of hemolymph and phagocyte filopodia in the cellular reactions. Dev. Comp. Immunol. 7, 199–208.

Wang, X.G., Rocheleau, T.A., Fuchs, J.F., Christensen, B.M., 2006. Beta 1, 3-glucan recognition protein from the mosquito, Armigeres subalbatus, is involved in the recognition of distinct types of bacteria in innate immune responses. Cell. Microbiol. 8, 1581–1590.

Wu, G.Q., Zhao, Z.Y., Liu, C.L., Qiu, L.H., 2014. Priming galleria mellonella (Lepidoptera: Pyralidae) larvae with heat-killed bacterial cells induced an enhanced immune protection against photorhabdus luminescens TT01 and the role of innate immunity in the process. J. Econ. Entomol. 107, 559–569.

Wu, G., Yi, Y., Lv, Y., Li, M., Wang, J., Qiu, L., 2015a. The lipopolysaccharide (LPS) of Photorhabdus luminescens TT01 can elicit dose- and time-dependent immune priming in Galleria mellonella larvae. J. Invertebr. Pathol. 127, 63–72.

Wu, G.Q., Li, M., Liu, Y., Ding, Y., Yi, Y.H., 2015b. The specificity of immune priming in silkworm, Bombyx mori, is mediated by the phagocytic ability of granular cells. J. Insect Physiol. 81, 60–68.

Wu, G.Q., Yi, Y.H., Sun, J.Y., Li, M., Qiu, L.H., 2015c. No evidence for priming response in Galleria mellonella larvae exposed to toxin protein PirA(2)B(2) from Photorhabdus luminescens TT01: an association with the inhibition of the host cellular immunity. Vaccine 33, 6307–6313.

Xia, Q., Zhou, Z., Lu, C., Cheng, D., Dai, F., Li, B., Zhao, P., Zha, X., Cheng, T., Chai, C., Pan, G., Xu, J., Liu, C., Lin, Y., Qian, J., Hou, Y., Wu, Z., Li, G., Pan, M., Li, C., Shen, Y., Lan, X., Yuan, L., Li, T., Xu, H., Yang, G., Wan, Y., Zhu, Y., Yu, M., Shen, W., Wu, D., Xiang, Z., Yu, J., Wang, J., Li, R., Shi, J., Li, H., Su, J., Wang, X., Zhang, Z., Wu, Q., Li, J., Zhang, Q., Wei, N., Sun, H., Dong, L., Liu, D., Zhao, S., Zhao, X., Meng, Q., Lan, F., Huang, X., Li, Y., Fang, L., Li, D., Sun, Y., Yang, Z., Huang, Y., Xi, Y., Qi, Q., He, D., Huang, H., Zhang, X., Wang, Z., Li, W., Cao, Y., Yu, Y., Yu, H., Ye, J., Chen, H., Zhou, Y., Liu, B., Ji, H., Li, S., Ni, P., Zhang, J., Zhang, Y., Zheng, H., Mao, B., Wang, W., Ye, C., Wong, G.K., Yang, H., 2004. A draft sequence for the genome of the domesticated silkworm (Bombyx mori). Science 306, 1937–1940.

Xiong, G.H., Xing, L.S., Lin, Z., Saha, T.T., Wang, C.S., Jiang, H.B., Zou, Z., 2015. High throughput profiling of the cotton bollworm Helicoverpa armigera immunotranscriptome during the fungal and bacterial infections. BMC Genomics 16.

Zhang, S.M., Adema, C.M., Kepler, T.B., Loker, E.S., 2004. Diversification of Ig superfamily genes in an invertebrate. Science 305, 251–254.

Zhang, Y.H., Jiang, R.X., Wu, H.S., Liu, P., Xie, J.Q., He, Y.Y., Pang, H., 2012. Next-generation sequencing-based transcriptome analysis of Cryptolaemus montrouzieri under insecticide stress reveals resistance-relevant genes in ladybirds. Genomics 100, 35–41.

Zhang, Z., Zhang, P., Li, W., Zhang, J., Huang, F., Yang, J., Bei, Y., Lu, Y., 2013. De novo transcriptome sequencing in Frankliniella occidentalis to identify genes involved in plant virus transmission and insecticide resistance. Genomics 101, 296–305.

Zhang, K., Pan, G.Z., Zhao, Y.Z., Hao, X.W., Li, C.Y., Shen, L., Zhang, R., Su, J.J., Cui, H.J., 2017. A novel immune-related gene HDD1 of silkworm Bombyx mori is involved in bacterial response. Mol. Immunol. 88, 106–115.

Zhao, Z., Wu, G., Wang, J., Liu, C., Qiu, L., 2013. Next-generation sequencing-based transcriptome analysis of Helicoverpa armigera larvae immune-primed with Photorhabdus luminescens TT01. PLoS One 8, e80146.

Zheng, X.L., Xia, Y.X., 2012. beta-1,3-Glucan recognition protein (beta GRP) is essential for resistance against fungal pathogen and opportunistic pathogenic gut bacteria in Locusta migratoria manilensis. Dev. Comp. Immunol. 36, 602–609.