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## RESEARCH ARTICLE - BEES

### Differential Gene Transcription in Honeybee (*Apis cerana*) Larvae Challenged by Chinese Sacbrood Virus (CSBV)

Y ZHANG<sup>1</sup>, X HUANG<sup>2,1</sup>, ZF XU<sup>2</sup>, RC HAN<sup>1</sup>, JH CHEN<sup>1</sup>

1 - Guangdong Entomological Institute, Guangzhou, Guangdong, China.

2 - South China Agricultural University, Guangzhou, Guangdong, China.

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#### Corresponding author

Richou Han

Guangdong Entomological Institute  
105 Xingang Road West, Guangzhou  
Guangdong, 510260, China  
E-Mail: richou-han@163.net

#### Abstract

Honey bees are economically important social insects. They are suffering from all kinds of pathogens, especially the viruses. In response to pathogens, different immune pathways such as Toll, Imd, Jak-Stat and RNAi are involved. In the present study, the transcription analysis of 32 immune-related genes from *Apis cerana* challenged by Chinese sacbrood virus (CSBV), the most widely distributed virus in *A. cerana*, was carried out by qRT-PCR to provide cues for the antiviral mechanism and the effective control of bee viruses. The expression level of 22 genes were statistically changed, including 11 up-regulated genes (*cactus-2*, *lys-2*, *vir*, *s3a*, *mta1*, *faa*, *vhd1*, *co-1-iv*, *ago-1*, *ago-3*, *aub*) in which 3 (*ago-1*, *ago-3*, *aub*) were related to RNAi pathway, and 11 down-regulated genes (*kenny*, *pgrp-lc*, *pgrp-s2*, *abaecin*, *lys-1*, *lys-3*, *domeless*, *tepa*, *mlc*, *dscam*, *rpl8*) related to Toll, Imd, and Jak-Stat pathways. The results indicated that CSBV infection in *A. cerana* may activate the whole immunity systems, including the RNA-based antiviral immunity system. This work constituted the first report, under laboratory conditions, about induction of immune related genes in response to CSBV.

#### Introduction

Insects have diverse mechanisms to combat infection by pathogens (Evans et al., 2006), including cellular and humoral immune defenses. The innate immune response of insect actions include the secretion of antimicrobial peptides, phagocytosis, melanization and the enzymatic degradation of pathogens (Hoffmann, 2003; Hultmark, 2003), which result from triggering the four non-autonomous pathways, Toll (transmembrane signal transducing pathway serves in both immunity and development), Imd (immune deficiency), JAK/STAT (the Janus kinase/signal transducers and activators of transcription) and JNK (intracellular signalling pathways) (Boutros et al., 2002). Viral double-stranded RNA is also recognized as a pathogen-associated molecular pattern and processed into small interfering RNAs (siRNAs) by the host ribonuclease Dicer. After amplification by host RNA-dependent RNA polymerases in some cases, these virus-derived siRNAs guide specific antiviral immunity through RNA interference and related RNA silencing effector mechanisms (Ding, 2010).

*Apis mellifera* and *Apis cerana* are the major honey bee species in the global beekeeping industry (Gallai et al., 2009; Garibaldi et al., 2011). They are heavily infected by different vital viruses (Chen et al., 2004, 2006; Ai et al., 2012). The *A. mellifera* genome (Honeybee Genome Sequencing Consortium, 2006) revealed that honey bees possess homologues of members of the four pathways implicated in humoral immune responses (Evans et al., 2006).

Chinese sacbrood virus (CSBV) is the most stricken pathogen of *A. cerana*, which results in severe and deadly infections of the colony and eventually losses of the entire colony (Yan et al., 2008; Liu et al., 2010; Han et al., 2013). This virus was first observed in Guangdong Province, China in 1972 and spread to the whole China and the counties of Southeast Asia (Liu et al., 2010). Some efforts have been made to study this virus, such as diagnostic methods (electron microscopy, enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction (RT-PCR)) (Yan et al., 2009), and the control of this disease by RNA interference (Liu et al., 2010). It is interesting that *A. mellifera* is not sensitive to CSBV in general beekeeping practice



(Zhang *et al.*, 2001). Furthermore, *A. mellifera* may develop cellular and humoral immune responses to various pathogens such as bacteria (Chan *et al.*, 2009; Lourenço *et al.*, 2013), viruses (Azzami *et al.*, 2012), microsporidian (Antúnez *et al.*, 2009; Chaimanee *et al.*, 2012) and *Varroa* mites (Gregorc *et al.*, 2012). However, no information on the expression of immune-related genes from *A. cerana* after infection of CSBV is reported.

In the present study, the transcription analysis of the immune-related genes from *A. cerana* challenged by CSBV was conducted to provide cues for the antiviral mechanism of the honey bees and the effective control of the bee viruses.

## Materials and Methods

### Honey Bees

Second instar larvae of *A. cerana* were obtained from a single mated honeybee queen in a healthy apiary at Conghua, Guangdong Province (113°17' E, 23°8' N), China. To obtain age controlled larvae, the queen was caged on a comb and left to lay eggs for six hours. Twenty hours after larval eclosion, or ninety-two hours after oviposition, the comb containing second instar larvae was retrieved from the colony, and placed in the laboratory for treatments at 32°C - 34°C. All the larvae used in this study were detected by RT-PCR method for the absence of the following viruses, black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), deformed wing virus (DWV), kashmir bee virus (KBV), CSBV and Israeli acute paralysis virus (IAPV), with the primers from Ai *et al.* (2012). RNA was extracted using Trizol Reagent (Invitrogen) and cDNA synthesis was performed using PrimeScript™ 1st Strand cDNA Synthesis Kit with DNA eraser (TaKaRa, Japan). Amplification profile of PCR consisted of an initial 2-min denaturation at 94 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C and finally 7-min cycle at 72 °C. No signs of clinical American foulbrood or viral disease were observed in these larvae.

### Virus

CSBV-infected larvae with typical symptom were collected from an apiary at Xinhua Village from Hunan Province, and kept at -80 °C for less than 10 days before use. The presence of CSBV virus was confirmed by observing the morphological symptom under electronic microscopy and RT-PCR according to Chen *et al.* (2004, 2006) and Liu *et al.* (2010). To obtain CSBV, the larvae (approximately 0.2 g) infected by CSBV were ground in 6 mL sterile phosphate-buffered solution (PBS) (1x: 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) with a sterile grinder. The resulting solution was centrifuged at 12,000 rpm at 4 °C for 10 min, and the supernatant was further passed through a 0.45 µm cell filter first, then through a 0.22 µm cell filter

(Ghosh *et al.*, 1999). CSBV concentration was quantitated by absolute quantification assay (Liu *et al.*, 2010). CSBV polyprotein gene (*sbv1*) was amplified to detect copy number of CSBV according to Liu *et al.* (2010). PCR reactions were carried out in triplicate in an Mx3000PTM Real-Time PCR System (Stratagene, California, USA), using SYBR\_Green (Brilliant II SYBR-Green QPCR Master Mix; Stratagene, California, USA). CSBV concentration for the experiments was  $7.20 \times 10^4$  copies/mL.

### Viral challenge and sample collection

Second instar larvae were collected from a comb with a special grafting tool and transferred to a 96-well tissue culture plate filled with 30 µL of basic diet prepared according to Patel *et al.* (2007). The larvae were maintained in an incubator (Sanyo, Tokyo, Japan) at 33°C - 34°C and 75% relative humidity. Twenty-four hours later, each larva was fed with 30 µL CSBV suspension or PBS for 24 h. Then, twenty larvae were picked from each replicates, sterilized with 75% ethanol (prepared with 0.2% Diethylpyrocarbonate (DEPC)-Water) for 5 min, washed with DEPC-Water for three times, immediately frozen in liquid nitrogen, and kept at -80°C until total RNA isolation (Liu *et al.*, 2010; Zhang *et al.*, 2010). Three replicates with 50 larvae in each were used for both treatments.

### Gene selection and primers

A total of 32 genes from *A. cerana* were selected for quantitative analysis according to references on the innate immune pathways in insects (Toll, Imd, JAK/STAT and RNAi pathways) (Table 1) (Christophides *et al.*, 2004; Clem *et al.*, 2005; Dostert *et al.*, 2005; Royet *et al.*, 2005; Yang & Cox-Foster, 2005; Evans *et al.*, 2006; Ding *et al.*, 2010). Primers and Genebank accession numbers of the corresponding genes was summarized in Table 1.

### Quantitative Real-time PCR (qRT-PCR)

Quantitative Real-Time PCR was carried out according to our previously described method with modifications (Zhang *et al.*, 2010). Briefly, total RNA and cDNA were prepared as described above. Expression of *actin* gene (GI: 406122) was used as an internal control. The efficiency of each primer set was first validated by constructing a standard curve through five serial dilutions. PCR reactions were carried out in triplicate in an Mx3000PTM Real-Time PCR System (Stratagene, USA), using SYBRGreen (SYBR® Premix Ex Taq™ II, TAKARA, Japan). A control without template was included in all batches. The PCR program began with a single cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 45 s. Afterwards, the PCR products were heated to 95 °C for 30 s, cooled to 55 °C for 30 s and heated to 95 °C for 30 s, in order to measure the dissociation curves

and to determine a unique PCR product for each gene. mRNA levels were calculated relative to *actin* expression using the Mx3000PTM Software (version 4.1) (Agilent, USA). The fold change was calculated using the  $2^{-\Delta\Delta C_t}$  method. Each sample was analyzed independently and processed in triplicate. PCR products were diagnosed by 1% agarose electrophoresis. Positive samples were cloned to pMD-19T (TAKARA, Japan), then sent to Invitrogen Company China for sequencing with corresponding specific primers.

### Data Analysis

Data were analyzed using SPSS statistical software (version 16.0) and the significance between treatments in each experiment was evaluated by One-Way ANOVA test followed by Tukey's test for pair comparisons at  $p < 0.05$ . The values are expressed as means  $\pm$  S.D. ( $p < 0.05$ ) was defined as statistically significant.

### Results

Bee colonies did not present any visible clinical symptoms of any disease (i.e., American Foulbrood or Chalkbrood). *Nosema* spp. spores were not detected from the bee samples. No CSBV, BQCV, CBPV, DWV, KBV, SBV, or IAPV viruses were detected in all the larvae used in this study. CSBV was only present in the larvae fed with CSBV.

RNA expression levels of 32 genes were determined using qRT-PCR 24 h after CSBV challenge. A set of 32 genes showed differential expression in the larvae challenged by CSBV. Gene-specific amplification was confirmed for 32 primer pairs by sequencing, by a single peak in melt-curve analysis and a single band with the expected size in agarose gel electrophoresis. No primer-dimer formation was detected and the standard curves derived from five-fold serial dilutions of pooled cDNA from the larvae (CSBV-challenged/control) gave correlation coefficients greater than 0.995 and efficiencies between 80% and 103%. Among 32 differential expression genes, 11 genes (*cactus-2*,  $P = 0.0269$ ; *lys-2*,  $P = 0.0173$ ; *vir*,  $P = 0.0451$ ; *s3a*,  $P = 0.0399$ ; *mtal*,  $P = 0.0219$ ; *faa*,  $P = 0.0233$ ; *vhdl*,  $P = 0.0400$ ; *co-1-iv*,  $P = 0.0141$ ; *ago-1*,  $P = 0.0018$ ; *ago-3*,  $P = 0.0186$ ; *aub*,  $P = 0.0142$ ) were up-regulated, 11 genes (*Kenny*,  $P = 0.0057$ ; *pgrp-lc*,  $P = 0.0418$ ; *pgrp-s2*,  $P = 0.0352$ ; *abaecin*,  $P = 0.0247$ ; *lys-1*,  $P = 0.0048$ ; *lys-3*,  $P = 0.0276$ ; *domeless*,  $P = 0.0434$ ; *tepa*,  $P = 0.0336$ ; *mhc*,  $P = 0.0389$ ; *dscam*,  $P = 0.0012$ ; *rp18*,  $P = 0.012$ ;) down-regulated, and 10 gene (*galectin*,  $P = 0.2860$ ; *cactus-1*,  $P = 0.1460$ ; *imd*,  $P = 0.0991$ ; *basket*,  $P = 0.1010$ ; *def2*,  $P = 0.1070$ ; *tpi*,  $P = 0.1200$ ; *ago-2*,  $P = 0.0765$ ; *dicer-1*,  $P = 0.0629$ ; *dicer*,  $P = 0.2570$ ; *piwi*,  $P = 1.9400$ ;) showed no significant difference in expression (ANOVA, Tukey's test,  $p < 0.05$ ) (Fig 1).

Of the up-regulated expression genes, *cactus-2*, *lys-2* were genes related to Toll pathway; *ago-1*, *ago-3*, and *aub* were members of RNAi pathways; *Vhdl* and *co-1-iv* were re-

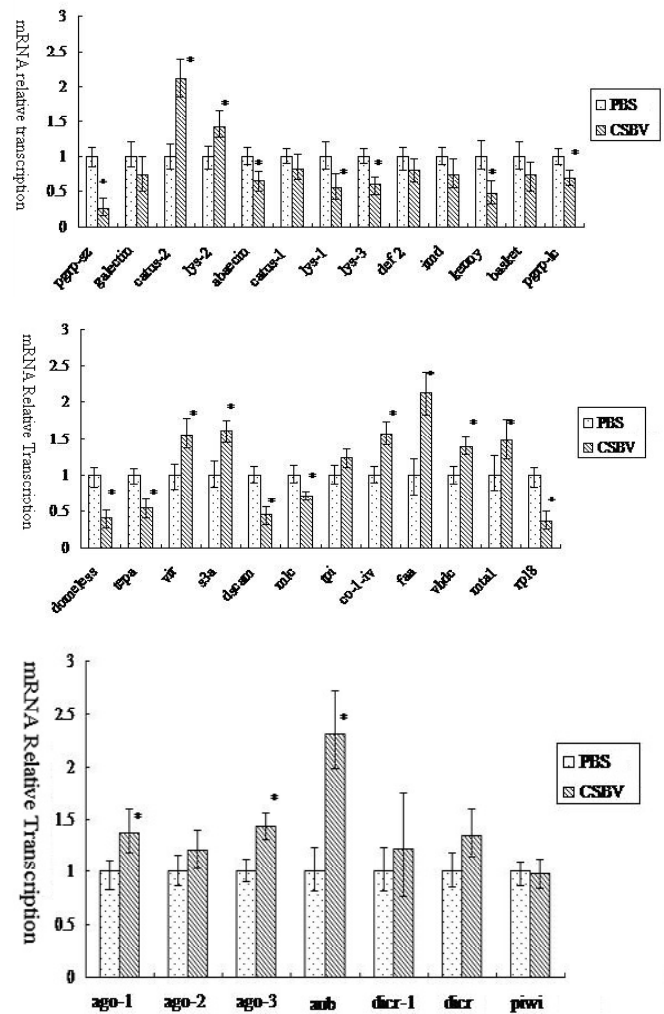


Fig 1 Relative transcription analysis of selected genes in CSBV-infected *Apis cerana* larvae by qRT-PCR. CSBV - The larvae fed with CSBV for 24 h. PBS - The larvae fed with phosphate-buffered solution as control. Results are reported as an average of the triplicates plus the standard deviation. Bars with different letters indicated significant differences by Tukey's test at  $P < 0.05$ (\*).

lated to cellular component and development; *mtal*, *faa*, and *s3a* were involved in cellular metabolic and regulated progress. Among the down-regulated genes, *kenny*, *pgrp-lc* were the important members in Imd pathways; Imd is specific for antimicrobial defense and normal development; *pgrp-s2* coding peptidoglycan recognition protein critically was involved in sensing bacterial infection and activation of the Toll signaling pathway (Evans et al., 2006); *Abaecin*, *lys-1*, *lys-2* and *lys-3* were antimicrobial effectors of Toll pathway; *domeless*, *vir* and *tepa* were the members of Jak pathway; *mhc* was related to cellular component and development; *Dscam* was related to humoral immunity (Evans et al., 2006); *rp18* was involved in cellular metabolism and regulation.

No selected genes specific to RNAi pathway displayed down-regulated expression, although *ago-2*, *dicer-1*, *dicer*, and *piwi* showed no significant difference in expression.

Table 1 - Locus names, gene annotation, primers and genebank accession numbers for the selected genes

Gene Name	Gene ID	Gene annotation	Primers	Regulation <sup>1</sup>	References <sup>2</sup>		Regulation
					References	host	
<i>galectin</i>	GB10026	cellular regulation	F: ACCACCTTCAGGTCCTGGTT R: CCCAGAGAGCCAAAGGTTTCC	D	-		
<i>co-1-iv</i>	GB14564	collagen	F: GGTACGTTTCGTTCCCGGTT R: TACCTTGCTCGCCCTGTAA	U	-		
<i>tpi</i>	gjl148224276	Glycolytic enzyme	F: CATGGGTAATTCCTGGTC R: CATGGGTAATTCCTGGTC	E	-		
<i>faa</i>	GB13982	hydrolase	F: AGGGATAATTTGGTTTGAATGG R: GTGGAGAAGATGCTCTGGGAT	U	Zhang et al., 2010	<i>A. cerana</i>	<i>Varroa destructor</i>
<i>pgrp-Ic</i>	GB17188	Imd pathway	F: TCCGTCAGCCGTAGTTTTTC R: CGTTTTGTGCAAAATCGAACAT	D	George et al., 2002	<i>Anopheles</i>	<i>E. coli</i>
<i>kenny</i>	GB17106	Imd pathway	F: GCTGAACCAGAAAGCCACTT R: TGC AAGTATGATGTGGA	D	Evans et al. et al., 2006	<i>A. mellifera</i>	<i>E. coli</i>
<i>imd</i>	GB18606	Imd pathway	F: TGTTAACGACCCGATGCAAAA R: CATCGCTCTTTTCGGATGTT	E	Evans et al. et al., 2006	<i>A. mellifera</i>	<i>P. larvae</i>
<i>basket</i>	GB16401	Imd pathway	F: AGGAGAACGTGGACATTTGG R: AATCCGATGGAAACAGAACG	E	Evans et al. et al., 2006	<i>A. mellifera</i>	<i>E. coli</i>
<i>dscan</i>	GB11871	Immunoglobulin	F: TTCAGTTCACAGCCCGAGATG R: ATCAGTGTCCCGTAAACCTG	D	Evans et al. et al., 2006	<i>A. mellifera</i>	<i>P. larvae</i>
<i>tepa</i>	GB18789	Jak pathway	F: CAAGAAGAAACGTGCGTGAA R: ATCGGGCAGTAAAGGACATTG	D	Evans et al., 2006	<i>A. mellifera</i>	<i>E. coli</i>
<i>domeless</i>	GB16422	Jak pathway	F: TTGTGCTCCTGAAAATGCTG R: AACCTCCAAATCGCTCTGTG	D	Xi et al., 2008	<i>Aedes aegypti</i>	<i>P. larvae</i>
<i>vir</i>	100872984	Jak pathway	F: GGGTAGTGTAGGCAAGAGGGG R: CTTGTGTCCAAAGGGCCGACTT	U	Dostert et al., 2005	<i>Drosophila</i>	(dengue virus serotype 2) DENV-2 Drosophila C Virus (DCV)
<i>vhdl</i>	GB726182	Lipoprotein	F: GCATCACCTTCTGACCAACC R: ACCTCGTCCAACATCCTTCT	U	Zhang et al., 2010	<i>A. cerana</i>	<i>Varroa</i>
<i>mlc</i>	GB409881	Myosin regulatory	F: AATCTCTTCGCATCTCCG R: CGCATCGTTGACTTCCCTT	D	Scharlaken et al., 2008	<i>A. mellifera</i>	<i>E. coli</i>

<sup>1</sup> Regulation in this study; <sup>2</sup> Regulation, challenged hosts and pathogens from the references; U: up-regulated genes; D: down-regulated genes; E: no differential expression genes.



(Continuation)

<i>mta1</i>	gj110756803	NURD	F: CATCTCTGTGCTTCTCCTC R: ACTCGATCTGGTTGTTTT	U			
<i>s3a</i>	gj166547340	Ribosoma lprotein	F: GTGCGGGTAGTAAGAAGCGAAGAAG R: GTGCGGGTAGTAAGAAGCGAAGAAG	U	-		
<i>rp18</i>	GB17629	Ribosoma lprotein	F: TGGATGTTCAACAGGGTTCATA R: CTGGTGGTGGACGTATTGATAA	D	-		
<i>ago-1</i>	GB122654	RNAi pathway	F: TGGCCACAGATCAAGTAGAGC R: AATTTGATAGCGTTTGTGGTGAT	U	Ding, 2010		
<i>ago-2</i>	GB15464	RNAi pathway	F: GAGCTATTGCGCGCTTAGAGA R: TATCAACAATGGTGCCCGCC	E	Ding, 2010	<i>Drosophila</i>	Flock house virus mutant (FHX)
<i>ago-3</i>	GB19389	RNAi pathway	F: CCCAATGCCCGATGTGATGT R: TCCATTCTGCAGGCTTTGCCC	U	Ding, 2010; Keene <i>et al.</i> , 2004	<i>Anopheles gambiae</i>	O'nyong-nyong virus mutant
<i>aub</i>	GB10293	RNAi pathway	F: TGGTTGGGATGTTTGGCCACA R: TCGCATAGCACCTCTTCCCA	U	Ding, 2010		
<i>dicer</i>	GB15170	RNAi pathway	F: AGCAGTAGCTGATGTGTGGA R: TTCAGAAAGCGCAAGGCATGT	U	Ding, 2010		
<i>dicer-1</i>	GB11966	RNAi pathway	F: AAGAGCTCCAGATGCCCTGT R: TGCATCACCTCCATCAAGTGG	E	Ding, 2010		
<i>piwi</i>	412427	RNAi pathway	F: TGCAAAAGAAACAGTGTGGA R: TCGCATAGCACCTCTTCCCA	E	Ding, 2010		
<i>abaecin</i>	GB18323	Toll pathway	F: CAGCATTCGCATACGTACCA R: GACCAGGAAACGTGGAAAC	D	Evans <i>et al.</i> , 2006	<i>A. mellifera</i>	<i>E. coli</i> U
<i>cactus-1</i>	GB10655	Toll pathway	F: CACAAGATCTGGAGCAACGA R: GCATTTTGAAGGAGGAACG	E	Evans <i>et al.</i> , 2006	<i>A. mellifera</i>	<i>P. larvae</i> U
<i>cactus-2</i>	GB13520	Toll pathway	F: TTAGCAGGACAAACGGCTCT R: CAGAAAAGTGGTTCGGGTGTT	U	Xi <i>et al.</i> , 2008	<i>Aedes aegypti</i>	<i>P. larvae</i> (dengue virus serotype 2) D
<i>def2</i>	GB10036	Toll pathway	F: GCAACTACCCGCTTTACGTC R: GGGTAACGTGGCAGCTTTTA	E	Evans <i>et al.</i> , 2006;	<i>A. mellifera</i>	DENV-2 <i>E. coli</i> U
<i>lys-1</i>	GB10231	Toll pathway	F: GAACACACGGTTGGTCACTG R: ATTTCCCAACCAATCGTTTTCC	D	Evans <i>et al.</i> , 2006	<i>A. mellifera</i>	<i>P. larvae</i> U
<i>lys-2</i>	GB15106	Toll pathway	F: CCAAATTAACAGCGCCAAGT R: GCAATTTCTCACCCCAACCAI	U	Evans <i>et al.</i> , 2006	<i>A. mellifera</i>	<i>P. larvae</i> D
<i>lys-3</i>	GB19988	Toll pathway	F: TCGATGAATGCGAAGAAATC R: TCGATGAATGCGAAGAAATC	D	Evans <i>et al.</i> , 2006	<i>A. mellifera</i>	<i>E. coli</i> U
<i>pgrp-s2</i>	GB19301	Toll pathway	F: TAATTCATCATTCGGCGACA R: TGTTTTGTCCCATCCTCTCC	D	Evans <i>et al.</i> , 2006	<i>A. mellifera</i>	<i>P. larvae</i> U
<i>actin</i>	GB406122	Reference gene	F: ATGCCAACACTGTCCTTTCTGG R: GACCCACCAATCCATACGGA	-			<i>E. coli</i> U <i>P. larvae</i>

## Discussion

As social animals, honey bees are at considerable risk from parasites and pathogens (Gregory et al., 2005; Yang & Cox-Foster, 2005; Evans, 2006; Azzami et al., 2012). Specifically, increased genetic relatedness and the high population densities of honey bee societies can strongly favour pathogen spread and epizootic outbreak. However, compared to the sequenced *Drosophila* and *Anopheles* genomes, honey bees are relatively immunologically deficient (i.e., express fewer immune response proteins) (Evans et al., 2006). Usually, in response to bacterial pathogens, fungal pathogens, parasite or acaricides, the expression level of antimicrobial peptides were up-regulated in the infected bees (Gregory et al., 2005; Evans, 2006; Scharlaken et al., 2008; Aronstein et al., 2010; Dussaubat et al., 2012; Gregorc et al., 2012; Hamiduzzaman et al., 2012; Garrido et al., 2013). The up-regulated genes were detected from *A. mellifera* after response to bacteria (*ERp60*, *obp17*, *proPO*, *HSPs*; *lys*, *hymenoptaecin*, *transferrin-labaecin*, *defensin*, *hymenoptaecin*, *pgrp-s3*, *B-glc-2*, *cactus-2*, *dorsal-1B*, *relish*) (Scharlaken et al., 2008; Chan et al., 2009; Lourenço et al., 2013), microsporidian (*abaecin*, *defensin*, *hymenoptaecin*) (Antúnez et al., 2009; Chaimanee et al., 2012) and *Varroa* mites (*pgrp-sc*, *abaecin*, *defensin1*, *hymenoptaecin*) (Gregorc et al., 2012). However, infection of *A. mellifera* with ABPV produced neither elevated levels of specific antimicrobial peptides (AMPs), such as hymenoptaecin and defensin, nor any general antimicrobial activity, such as nodulation (Azzami et al., 2012). These data suggest that bees use a distinct mechanism to counter different viral infections. The present results showed that *A. cerana* produced expression changes of immune-related genes when responding to CSBV infection. RNAi based antiviral pathway might be very important for *A. cerana*.

In the present study, after challenged by CSBV, *A. cerana* larvae produced 3 down-regulated (*pgrp-s2*, *abaecin*, *lys-1*) and 2 up-regulated (*cactus-2*, *lys-2*) genes for Toll pathway. However, in response to *Paenibacillus larvae*, the genes (*pgrp-s2*, *abaecin*, *lys-1* and *lys-3*) in *A. mellifera* were strongly up-regulated, while *cactus-2* and *lys-2* were down-regulated (Evans et al., 2006). The differences in gene expression patterns of Toll pathway may be related with the bee species and responding pathogens. Moreover, from the gene regulation pattern, to a certain extent, it seemed that the Toll pathway was suppressed by CSBV infection.

Of 4 genes (*imd*, *basket*, *kenny*, *pgrp-lc*) related to Imd pathway, 2 genes (*kenny* and *pgrp-lc*) were down-regulated and others (*Imd* and *basket*) showed no significant difference in expression. PGRP-LC is the activator of the Imd signaling process, which elicited by peptidoglycan, fungi and *Varroa* mites (Werner et al., 2003; Stenbak et al., 2004; Gregorc et al., 2012). It was apparent that CSBV infection suppressed the expression of *pgrp-lc*.

Among 3 genes related to Jak/STAT pathway, 2 genes

(*domeless*, *tepa*) were up-regulated, but *vir* (virus-induced RNA) was down-regulated. *Vir* was not induced by pathogenic bacteria or fungi in *Drosophila* (Dostert et al., 2005). But Jak kinase Hopscotch was involved in the control of the viral load in infected flies (Dostert et al., 2005).

Previous studies also indicated that RNA interference as an intrinsic defense against viral infection play a major role in antiviral immunity in insects, e.g. *D. melanogaster*, *Anopheles gambiae*, *Bombyx mori*, *Caenorhabditis elegans* (Keene et al., 2004; Galiana-Arnoux et al., 2006, 2007; van RiJ. et al., 2006; Kemp & Imler, 2009; Azzami et al., 2012; Xu et al., 2012). Central to the RNAi mechanism are the slicing enzymes of the Argonaute (AGO) family (five members in *Drosophila*), which mediate highly specific cleavage of target RNA molecules. The specificity of AGO enzymes is achieved by their association with small RNAs, which guide them to complementary sequences (Ding, 2010). Three RNAi pathways, involving different members of the AGO family (AGO-1, AGO-2, AGO-3), have been defined in *Drosophila* (Kemp & Imler, 2009). In RNA silencing, AGO proteins are the effector molecules of specific gene silencing, the specificity of which is determined by the AGO-bound siRNA (Kemp & Imler, 2009; Ding, 2010). In our results, *ago-1*, *aub*, and *ago-3* in *A. cerana* larvae were significantly up-regulated after CSBV infection, although *ago-2*, *dicer-1*, *dicer* and *piwi* showed no significant expression difference, indicating that in honey bees there may be a RNA-based antiviral immunity system, though it can activate the whole immunity systems. Future studies are of importance to reveal the specific role of RNAi in the antiviral response of honey bees.

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## Reference

- Ai, H.X., Yan, X., & Han, R.C. (2012). Occurrence and prevalence of seven bee viruses in *Apis mellifera* and *Apis cerana* apiaries in China. *J. Invertebr. Pathol.*, 109: 160-164. doi: org/10.1016/j.jip.2011.10.006
- Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., & Higes, M. (2009). Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). *Environ. Microbiol.*, 11: 2284 - 90. doi: 10.1111/j.1462-2920.2009.01953.x

- Aronstein, K.A., Murray, K.D., & Saldivar, E. (2010). Transcriptional responses in honey bee larvae infected with chalkbrood fungus. *BMC Genomics*, 11: 391. doi: 10.1186/1471-2164-11-391.
- Azzami K., Ritter, W., Tautz, J., & Beier, H. (2012). Infection of honey bees with acute bee paralysis virus does not trigger humoral or cellular immune responses. *Arch. Virol.*, 157: 689 - 702. doi: 10.1007/s00705-012-1223-0
- Boutros, M., Agaisse, H. & Perrimon, N. (2002) Sequential activation of signaling pathways during innate immune responses in *Drosophila*. *Dev. Cell*, 3: 711 - 722. doi: org/10.1016/S1534-5807(02)00325-8
- Chaimanee, V., Chantawannakul, P., Chen, Y., Evans, J.D., & Pettis, J.S. (2012) Differential expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema ceranae*. *J. Insect Physiol.* 58: 1090 - 1095. doi: org/10.1016/j.jinsphys.2012.04.016
- Chan, Q.W., Melathopoulos, A.P., Pernal, S.F., & Foster, L.J. (2009). The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larvae*. *BMC Genomics*, 10: 387. doi:10.1186/1471-2164-10-387
- Chen, Y., Zhao, Y., Hammond, J., Hsu, H.T., Evans, J., & Feldlaufer, M. (2004). Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *J. Invertebr. Pathol.*, 87: 84 - 93. doi: org/10.1016/j.jip.2004.07.005
- Chen, Y.P., Pettis, J.S., Collins, A., & Feldlaufer, M.F. (2006). Prevalence and transmission of honeybee viruses. *Appl. Environm. Microbiol.*, 72: 606 - 611. doi: 10.1128/AEM.72.1.606-611.2006
- Christophides, G.K., Vlachou, D., & Kafatos, F.C. (2004). Comparative and functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. *Immunol. Rev.*, 198: 127 - 148. doi: 10.1111/j.0105-2896.2004.0127.x
- Clem, R.J. (2005). The role of apoptosis in defense against baculovirus infection in insects. *Curr. Top. Microbiol. Immunol.*, 289: 113 - 29. doi: 10.1007/3-540-27320-4\_5
- Ding, S.W. (2010). RNA-based antiviral immunity. *Nat. Rev. Immunol.*, 10: 632 - 644. doi:10.1038/nri2824
- Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J.A., & Imler, J.L. (2005). The jak-stat signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nat. Immunol.*, 6: 946 - 953. doi:10.1038/ni1237
- Dussaubat, C., Brunet, J.L., Higes, M., Colbourne, J.K., & Lopez, J. (2012). Gut pathology and responses to the microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*. *PLoS ONE* 7(5): e37017. doi:10.1371/journal.pone.0037017
- Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., & Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol.*, 15: 645 - 656. doi: 10.1111/j.1365-2583.2006.00682.x
- Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J.A., & Imler, J.L. (2006). Essential function in vivo for dicer-2 in host defense against RNA viruses in drosophila. *Nat. Immunol.*, 7: 590 - 597. doi:10.1038/ni1335
- Galiana-Arnoux, D., Deddouche, S., & Imler, J.L. (2007). Antiviral immunity in drosophila. *J. Soc. Biol.*, 201: 359 - 365. doi: org/10.1016/j.coi.2009.01.007
- Gallai, N., Salles, J.M., Settele, J., & Vaissière, B.E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol. Econ.*, 68: 810 - 821. doi: org/10.1016/j.ecolecon.2008.06.014
- Garibaldi, L.A., Steffan-Dewenter, I., Kremen, C., Morales, J.M., Bommarco, R., Cunningham, S.A., Carvalheiro, L.G., Chacoff, N.P., Dudenhoffer, J.H., Greenleaf, S.S., Holzschuh, A., Isaacs, R., Krewenka, K., Mandelik, Y., Mayfield, M.M., Morandin, L.A., Potts, S.G., Ricketts, T.H., Szentgyorgyi, H., Viana, B.F., Westphal, C., Winfree, R., & Klein, A.M. (2011). Stability of pollination services decreases with isolation from natural areas despite honey bee visits. *Ecol. Lett.*, 14: 1062-1072. doi: 10.1111/j.1461-0248.2011.01669.x
- Garrido, P.M., Antúnez, K., Martín, M., Porrini, M.P., Zunino, P., & Eguaras, M.J. (2013). Immune-related gene expression in nurse honey bees (*Apis mellifera*) exposed to synthetic acaricides. *J. Insect Physiol.*, 59: 113-119. doi: org/10.1016/j.jinsphys.2012.10.019
- Ghosh, R.C., Ball, B.V., Willcocks, M.M., & Carter, M.J. (1999). The nucleotide sequence of sacbrood virus of the honey bee: an insect picorna-like virus. *J. Gen. Virol.*, 80: 1541-1549.
- Gorg, A., Postel, W., Domscheit, A., & Günther, S. (1988). Two-dimensional electrophoresis with immobilized pH gradients of leaf proteins from barley (*Hordeum vulgare*): method, reproducibility and genetic aspects. *Electrophoresis*. 9: 681- 692.
- Gregorc, A., Evans, J.D., Scharf, M., & Ellis, J.D. (2012). Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and Varroa mites (*Varroa destructor*). *J. Insect Physiol.*, 58: 1042-1049. doi: org/10.1016/j.jinsphys.2012.03.015
- Gregory, P.G., Evans, J.D., Rinderer, T. & De Guzman, L. (2005) Conditional immune-gene suppression of honeybees parasitized by Varroa mites. *J. Insect Sci.*, 5: 1 - 5.
- Hamiduzzaman, M.M., Sinia, A., Guzman-Novoa, E., Guzman-Novoa, E., & Goodwin, P.H. (2012). Entomopathogenic fungi as potential biocontrol agents of the ecto-parasitic mite, *Varroa destructor*, and their effect on the immune response of honey bees (*Apis mellifera* L.). *J. Invertebr. Pathol.*, 111: 237 - 243.

- doi: org/10.1016/j.jip.2012.09.001
- Han, B., Zhang, L., Feng, M., Fang, Y., & Li, J.K. (2013). An integrated proteomics reveals pathological mechanism of honeybee (*Apis cerana*) sacbrood disease. *J. Proteome Res.*, 12: 1881-1897. doi: 10.1021/pr301226d
- Hoffmann, J.A. (2003). The immune response of *Drosophila*. *Nature*, 426: 33 - 38. doi:10.1038/nature02021
- Honey Bee Genome Sequencing Consortium. (2006). Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature*, 443: 931 - 949. doi: 10.1038/nature05260
- Hultmark, D. (2003). *Drosophila* immunity: Paths and patterns. *Curr. Opin. Immunol.*, 15: 12 - 19. doi: org/10.1016/S0952-7915(02)00005-5
- Keene, K.M., Foy, B.D., Sanchez-Vargas, I., Beaty, B.J., Blair, C.D., & Olson, K.E. (2004). RNA interference acts as a natural antiviral response to o'nyong-nyong virus (alphavirus; Togaviridae) infection of anopheles gambiae. *Proc. Natl. Acad. Sci. USA*, 101: 17240 - 17245. doi: 10.1073/pnas.0406983101
- Kemp, C., & Imler, J.L. (2009). Antiviral immunity in *Drosophila*. *Curr. Opin. Immunol.*, 21: 3 - 9. doi: org/10.1016/j.coi.2009.01.007
- Liu, X.J., Zhang, Y., Yan, X., & Han, R.C. (2010). Prevention of chinese sacbrood virus infection in *Apis cerana* using RNA interference. *Curr. Microbiol.*, 61: 422 - 428. doi: 10.1007/s00284-010-9633-2
- Lourenço, A.P., Guidugli-Lazzarini, K.R., Freitas, F.C., Bitondi, M.M., Simões, Z.L., Parker, R., Guarna, M.M., Melathopoulos, A.P., Moon, K.M., White, R., Huxter, E., Pernal, S.F., & Foster, L.J. (2013). Bacterial infection activates the immune system response and dysregulates microRNA expression in honey bees. *Insect Biochem. Mol. Biol.*, 43: 474 - 482. doi: org/10.1016/j.ibmb.2013.03.001
- Ma, M.X., Li, M., Cheng, J., Yang, S., Wang, S.D., & Li, P.F. (2011). Molecular and biological characterization of Chinese sacbrood virus LN isolate. *Comp. Funct. Genomics*, doi:10.1155/2011/409386
- Patel, A., Fondrk, M.K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., & Amdam, G.V. (2007). The making of a queen: TOR pathway is a key player in diphenic caste development. *PLoS ONE*, 2: e509. doi: 10.1371/journal.pone.0000509
- Royet, J., Reichhart, J.-M., & HoVmamm, J.A. (2005). Sensing and signaling during infection in *Drosophila*. *Curr. Opin. Immunol.*, 17: 11 - 17. doi: org/10.1016/j.coi.2004.12.002
- Scharlaken, B., de Graaf, D.C., & Goossens, K. (2008). Differential gene expression in the honeybee head after a bacterial challenge. *Dev. Comp. Immunol.*, 32: 883 - 889. doi: org/10.1016/j.dci.2008.01.010
- Stenbak, C.R., Ryu, J.H., Leulier, F., Pili-Floury, S., Parquet, C., Herve, M., Chaput, C., Boneca, I.G., Lee, W.J., Lemaitre, B. & Mengin-Lecreulx, D. (2004). Peptidoglycan molecular requirements allowing detection by the *Drosophila* immune deficiency pathway. *J. Immunol.*, 173: 7339 - 7348. Retrieved from: <http://www.jimmunol.org/content/173/12/7339.short>
- van RiJ, R.P., Saleh, M.C., Berry, B., Foo, C., Houk, A., Antoniewski, C., & Andino, R. (2006). The RNA silencing endonuclease argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster*. *Genes Dev.*, 20: 2985 - 2995. doi: 10.1101/gad.1482006
- Werner, T., Borge-Renberg, K., Hultmark, D., Mellroth, P. & Steiner, H. (2003). Functional diversity of the *Drosophila* PGRP-LC gene cluster in the response to lipopolysaccharide and peptidoglycan. *J. Biol. Chem.*, 278: 26319 - 26322. doi: 10.1074/jbc.C300184200
- Xu, Y., Huang, L., Fu, S., Wu, J., & Zhou, X. (2012). Population diversity of rice stripe virus-derived siRNAs in three different hosts and RNAi-based antiviral immunity in *Laodelphax striatellus*. *PLoS One*, 7: e46238. doi: 10.1371/journal.pone.0046238
- Yan, X., Chen, J.H., & Han, R.C. (2009). Detection of Chinese sacbrood virus (CSBV) in *Apis cerana* by RT-PCR method. *Sociobiology*, 53: 687 - 694.
- Yan, X., & Han, R.C. (2008). Diagnostic technologies of common pathogens of honeybees in China. *Chin. Bull. Entomol.*, 45: 483 - 488. Retrieved from: [http://en.cnki.com.cn/Article\\_en/CJFDTOTAL-KCZS200803039.htm](http://en.cnki.com.cn/Article_en/CJFDTOTAL-KCZS200803039.htm)
- Yang, X.L., & Cox-Foster, D.L. (2005). Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proc. Natl. Acad. Sci. USA*, 102: 7470 - 7475. doi: 10.1073/pnas.0501860102
- Zhang, Y., Liu, X.J., Zhang, W.Q., & Han, R.C. (2010). Differential gene expression of the honey bees *Apis mellifera* and *A. cerana* induced by *Varroa destructor* infection. *J. Insect Physiol.*, 56: 1207 - 1218. doi: org/10.1016/j.jinsphys.2010.03.019

\* Y ZHANG, X HUANG and RC HAN contributed equally to this work.