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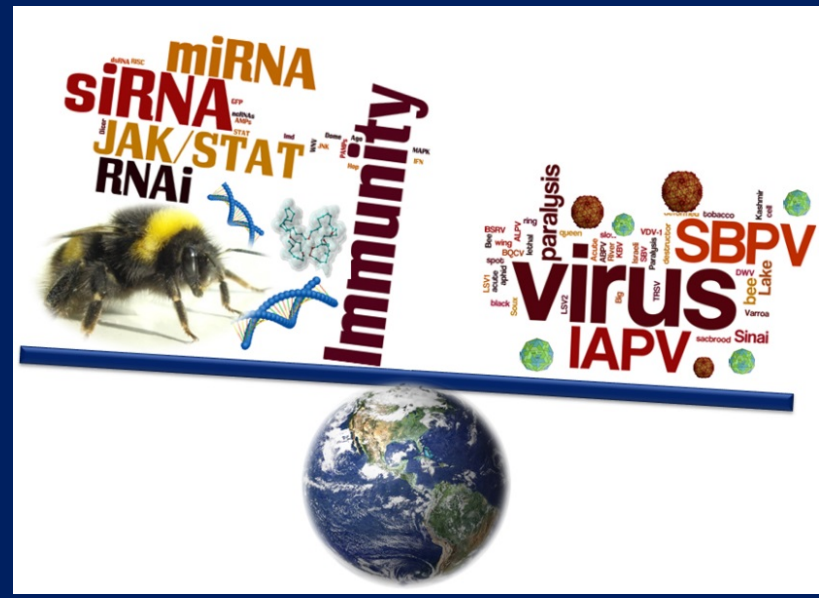

Jinzhi Niu

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UNIVERSITEIT GENT

FACULTY OF BIOSCIENCE ENGINEERING

In vivo study of RNA interference, the JAK/STAT pathway and their cross-talk in *Bombus terrestris* upon viral infections

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Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) in Applied Biological Sciences

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Front cover:

Top left: *Bombus terrestris* with the word cloud of host immunity associated key words, protein and DNA structures. Top right: virus structures with the word cloud of bee virus related key words. Bottom: a figure of earth and line represent the balance scale.

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Table of Contents

List of abbreviations

Abbreviation	Name
ABPV	acute bee paralysis virus
Ago	Argonaute
ALPV	aphid lethal paralysis virus
AMPs	Antimicrobial peptides
BQCV	black queen cell virus
BSRV	Big Soux River virus
CrPV	Cricket paralysis virus
DCV	Drosophila C virus
DENV	dengue virus
Dome	domeless
dpi	days post injection
dsRNA	double-stranded RNA
DWV	deformed wing virus
EGF	epidermal growth factor
ELF1α	elongation factor-1 α
ES	Elution buffer
gcn	genome copy number
GFP	green fluorescent protein
GO	gene ontology
Hop	tyrosine-protein kinase JAK2
IAPV	Israeli acute paralysis virus
IFN	interferon
Imd	immune deficiency
IRES	internal ribosome entry site
JAK/STAT	janus kinase/signal transducer and activator of transcription
JH	juvenile hormone
JNK	c-Jun N-terminal kinase
KBV	Kashmir bee virus
KEGG	Kyoto Encyclopedia of Genes and Genomes
LSV1	Lake Sinai virus 1
LSV2	Lake Sinai virus 2
MAPK	mitogen-activated protein kinases
miRNA	microRNA
ncRNAs	non-coding RNAs

Abbreviation	Name
NF-κB	nuclear factor κB
ORF	open reading frame
PAMPs	pathogen associated molecular patterns
PBS	10 mM phosphate buffer (pH 7.0)/0.02% diethyl dithiocarbamate
PPIA	peptidylprolyl isomerase A
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
RPL23	60S ribosomal protein
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
SBPV	slow bee paralysis virus
SBV	sacbrood virus
Sid	systemic RNA interference–deficient
SINV	Sindbis virus
SINV-GFP	Sindbis virus with green fluorescent protein
siRNA	small interfering RNA
sRNAs	small RNAs
STAT	signal transducer and activator of transcription
TBP	TATA-binding protein
TEM	transmission electron microscopy
TEP7	thioester-containing protein 7
TEPA	thioester-containing protein A
TEPB	thioester-containing protein B
TRSV	tobacco ring spot virus
UBI	polyubiquitin
Upd	ligand unpaired
UTR	untranslated region
VDV-1	*Varroa destructor* virus-1
vir-1	virus induced gene 1
vsiRNA	virus-derived small interfering RNAs
VSR	viral suppressors of RNAi
VWC	von Willebrand factor C-domain
WNV	West Nile virus

Scope

In super model insects, like *Drosophila* and the mosquito, the interactions between hosts and viruses are relatively well-described. However, this kind of knowledge is still very limited in bumblebees. With the recent release of two genomes from *Bombus terrestris* and *B. impatiens*, the research of bumblebees also steps into the genomic era. Although honeybees, bumblebees, and solitary bees, range from eusocial to solitary, they all have a relatively same small immune repertoire compared with other insects. The unique genetic aspects of the bee immune repertoire would be interesting to study in order to improve our basic understanding in insect immune evolution. Currently, scientists are trying to understand the reasons for bee colony collapse in the association with viral infections. The study on the antiviral immunity in the bees upon infection of different viruses may be useful to search for efficient measures to tackle the current problem in bees. In this dissertation we focused on the antiviral immunity upon two bee viruses, i.e. Israeli acute paralysis virus (IAPV) and slow bee paralysis virus (SBPV). Secondly, we chose IAPV and SBPV because: 1) both naturally infect bumblebees in the field; 2) each represents a key bee virus family, i.e. *Dicistroviridae* and *Iflaviridae*; 3) In our experimental setup, IAPV induced an extremely virulent infection while SBPV did not induce mortality of bees. We chose three important and possibly interacting pathways, namely the small interfering RNA (siRNA), microRNA (miRNA) and JAK/STAT (janus kinase/signal transducer and activator of transcription), to investigate immune responses of bees in the defense against viral infections.

The siRNA pathway is the most studied pathway with antiviral activity in bees and its usefulness to tackle bee virus-related diseases in the field is promising. However, the involvement of the

siRNA pathway to defend against different bee viruses is still poorly understood. Therefore, in **Chapter II**, we comparatively analyzed the siRNA antiviral response after inducing systemic infections of IAPV and SBPV in bumblebees based on three approaches: 1) analysis of core gene expressions of the siRNA upon virus infection; 2) deep sequencing to analyze small RNAs in virus infected samples; 3) using RNAi (RNA interference) to check the virus infection after silencing one of the core genes of the siRNA pathway, *Dicer-2*.

The miRNA pathway, a closely related pathway to the siRNA pathway, is also established to be involved in insect-pathogen interactions. In virus-infected hosts the miRNAs can have two origins, either encoded by the host or the virus. Both origins of miRNAs may target host genes and/or the viral RNA (genome), which results in a complex network of host-virus interactions based on miRNAs. In **Chapter III**, we aimed to generate more insight in the involvement of the miRNA pathway and bumblebee-encoded miRNAs upon viral infections. We performed a comparative analysis of the bumblebee miRNA pathway, upon infections of IAPV and SBPV. First, we screened the expression of core genes (*Dicer-1* and *Ago-1*) of the miRNA pathway upon viral infections; secondly, through small RNA sequencing, we analyzed the miRNA transcriptomes with viral infections. To have a further insight in miRNA-mRNA interaction, we predicted the possible targets for these miRNAs. Finally, we silenced *Dicer-1* to analyze the outcome of SBPV infection.

Next to nucleotides-based RNAi (including siRNA and miRNA pathways) immunity, another conserved protein-based pathway in insects, JAK/STAT, not only contributes to antiviral activity, but also represents a cross-talk with the siRNA pathway. In **Chapter IV**, we studied the role of the bumblebee JAK/STAT pathway in the control of IAPV and SBPV through silencing *Hop*, a key component of JAK/STAT pathway. Two different viruses with different virulence also gave

us the opportunity to study the immune cross-talk between the siRNA pathway and JAK/STAT. Through screening key gene expressions, we mainly focused on *Dicer-2* as a proxy for the siRNA pathway, *Vago* as a proxy for the cross-talk between the siRNA pathway and JAK/STAT pathway, and *Hop* as a proxy for JAK/STAT pathways, upon various treatments.

In the final **Chapter V** we integrated and discussed some perspectives in the links among the siRNA, miRNA, and JAK/STAT pathways. We emphasized on what our current results could imply for bee health upon bee virus infection and discussed possible research directions and applications.

Chapter I-General introduction

Parts of this chapter are published in:

Niu J, Meeus I, Cappelle K, Piot N,Smagghe G: The immune response of the small interfering RNA pathway in the defense against bee viruses. *Current Opinion in Insect Science* 2014, 6:22-27.

1. Bumblebee

1.1. Bumblebee colony

The common name, bumblebee, refers to a bee which bumbles, meaning making a buzzing or humming sound. Compared with the honeybee, bumblebees generally have larger, fuzzier, stouter bodies. When flying, they make a more noticeable sound and an energy sound (sonication) to expel pollen from flowers evolved to only release pollen after buzz pollination. They also have fewer stripes, large parts of the body covered in black fur and have lower provision of nectar/honey compared with the sister genus honeybees. Being most successful in temperate regions, bumblebees are natively found around the globe except for sub-Saharan Africa and Oceania. There are about 250 species of bumblebees recorded, which belong to the genus *Bombus*. Some species are social parasites taking advantage of the nest of other bumblebees and were originally classified as a separate genus *Psythirus,* but are currently classified as one of the many subgenera of *Bombus*. Although there is a big group of bumblebee species reported, studies have only focused on a handful of species, particularly *Bombus terrestris*, *B. impatiens*, and *B. ignitus*. Most of bumblebee species, exhibit an annual colony cycle (Figure 1) (Sadd et al., 2015). Unlike honeybees, bumblebees pass through several phases from solitary to eusocial during their life cycle. Thus, the study of bumblebee species can provide profound insights in the evolution of social behavior (Amsalem et al., 2015).

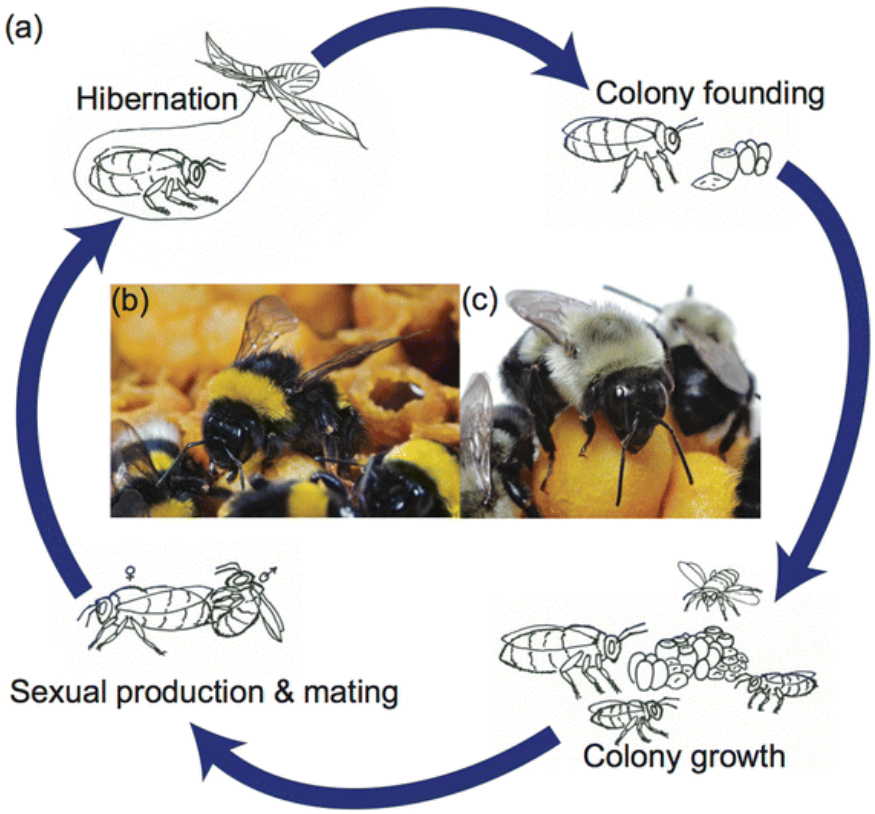


Figure 1: An illustrative colony cycle of bumblebee species living in temperate regions (Sadd et al., 2015)

(a). This is representative for the colony cycles of the two species, *Bombus terrestris* (b) and *B. impatiens* (c). Queen bumblebees emerge from hibernation, establish a nest as a single foundress and provision it with pollen and nectar. Egg batches are laid that develop into female worker offspring. Once these offspring have developed and emerged as adults they take over foraging duties from the queen, and tend to developing brood. After sustained colony growth, males and new queens are produced. These sexuals leave the colony and mate, following which the new queens hibernate while males and the remainder of the colony perishes. (Sadd et al., 2015)

Bumblebees generally visit flowers that exhibit the bee pollination syndrome to collect nectar and pollen, thus they are very important in pollination for crops and wildflowers. In recent decades, some species of bumblebee, such as *B. terrestris* and *B. impatiens*, have been commercially reared and distributed internationally for crop pollination, mainly for greenhouse crops. The honeybee, a highly advanced eusocial insect, has been intensively studied in various aspects, while the research on bumblebees is still relatively far behind. For instance, a web of

science search using the keyword "honeybee", "honeybee", or "*Apis mellifera*" resulted in 10,128, 12,056, or 12,071 hits, respectively. However using "bumblebee", "bumble bee" or "*Bombus terrestris*" it obtained 2,765, 1,775, or 1,432, respectively (accessed on September 9, 2015: http://apps.webofknowledge.com). Certainly, the studies from honeybee provide a good basis to study bumblebee as they share some similarities. When specially looking at differences of bumblebees compared with honeybees (Table 1) (Sadd et al., 2015), it forms an exciting case. Especially, with regard to the eusocial spectrum, bumblebees are an outstanding model system since they are in an intermediate position on the eusocial spectrum. The evolution of bee eusociality may arise from multiple independent transitions (Kapheim et al., 2015).

Table 1: Key differences and similarities between honeybees and the bumblebees. (Sadd et al., 2015)

	Honeybee	Bumblebees	
	Apis mellifera	*Bombus impatiens*	*Bombus terrestris*
Native range	Africa/Asia/Europe	Temperate North America	Palaearctic region
Nesting	Cavity nesters		
Nest location	Trees	Ground	
Foraging	Generalist foragers of nectar and pollen		
Colony cycle	Perennial	Annual with queen diapause	
Colony founding	Colony fission	Solitary nest founding	
Sociality	Advanced eusocial	Primitively eusocial	
Colony size	Approximately 20,000-100,000 workers	<400 workers	
Queen mating system	Highly polyandrous	Limited polyandry	Monandrous
Worker division of labor	Age-based	Some size- and age-based	
Caste differentiation	Morphology/Size/Physiology	Size/Physiology	
Worker reproduction	Rare	Common	
Human links	Managed (hundreds thousands of years)	Managed (decades)	

1.2. Bumblebee genome

Recently, two genomes of bumblebees, *B. terrestris* (249 Mb, Bter_1.0, accession AELG00000000.1) and *B. impatiens* (248 Mb, BIMP_2.0, accession AEQM00000000.2), have been sequenced. This makes bumblebee research enter a genomic era and would greatly improve the molecular study on these species. Some key features of these two genomes are listed by Sadd et al. (Sadd et al., 2015):

- The two bumblebee genomes exhibit extensive synteny, with limited rearrangements over the estimated 18 million years of divergence between the two lineages.
- Relatively few repetitive elements and a low diversity of transposable elements are presented, although there is some evidence of recent activity.
- Orthology and protein domain analysis uncover bee- and bumblebee-specific genes and domains, with hints of evolutionary processes differentially acting upon aspects relating to chemosensation and muscle function in the bumblebee lineage.
- *B. terrestris* and *B. impatiens* are extremely similar in terms of gene content related to developmental pathways in molting, metamorphosis, and exoskeleton dynamics. This gene repertoire shows striking similarities among social and non-social Hymenoptera.
- A similar set of genes underlying haplo-diploid sex determination is present relative to honeybees, despite an alternative primary signal for sex determination being employed.
- Genes associated with behavior, neurophysiology, and endocrinology are broadly conserved between *A. mellifera* and bumblebees, yet limited differences do exist, and in particular among Juvenile Hormone Binding Proteins this may be connected to functional differences between these species.
- Xenobiotic detoxifying enzymes present to be depauperate, as in *A. mellifera*, which has consequences for the ability of these species to deal with novel environmental xenobiotics, such as insecticides.
- Genes involved in chemoreception show expected complex patterns of gene birth and death. However, surprisingly, the gene repertoire of *B. terrestris* suggests that, relative to honeybees, bumblebees emphasize gustation over olfaction.
- Venom constituents, in general, are highly similar between honeybees and bumblebees.
- While components of all major immune pathways are present, as in *A. mellifera*, the complement of immune genes in the bumblebees is much reduced relative to Dipteran models, suggesting this is not a honeybee-specific characteristic, nor is it linked to advanced eusociality. Rather, it is likely that a reduced immune repertoire is basal to the bee lineage.
- RNAi core genes, RNA editing, and DNA methylation genes and genome wide patterns are highly conserved between *A. mellifera* and the two bumblebees.
- MicroRNAs (miRNAs) show a distinct pattern between the bumblebees and honeybees. Unique miRNAs were identified in both groups as well as potentially functionally

relevant changes in conserved miRNAs. These are excellent candidates that may tune key biological differences between advanced eusocial honeybees and primitively eusocial bumblebees.

2. Bee viruses

2.1. Bee virus species

The spreading of viral diseases is one of the suspects responsible for decline of bee colonies (Cox-Foster et al., 2007; Fürst et al., 2014). Under natural conditions, bee viruses are found in an array of wild and domesticated pollinators, forming an intricate multi-host network where the viruses can be transmitted among the different pollinators (Fürst et al., 2014; Levitt et al., 2013; Singh et al., 2010). The transmission pre-dominantly occurs due to common food sources, such as pollen and nectar, shared by the pollinator community. Moreover, multiple virus infections are also present in bees, specially honeybees and bumblebees, a number of up to 3-4 viruses can infect a single bee (De Smet et al., 2012; McMahon et al., 2015; Singh et al., 2010). These complex characteristics of viral infections challenge the bee's innate immune system. In addition, stressors like insecticides and *Varroa* mites (a viral vector for honeybees), could also affect the immune response of the bee, facilitating viral infection (Di Prisco et al., 2013; Francis et al., 2013; Ryabov et al., 2014). To date, there are around 23 viruses, the number varies based on different categories reported to infect bees worldwide. Mainly, they are from the families of *Dicistroviridae* and *Iflaviridae* in the order of *Picornavirales*. These viruses are non-enveloped small icosahedral virions, covering a positive sense single stranded RNA genome. Their genomes are around ~9 kb and contain one (in case of the *Iflaviridae*) or two (in case of the *Dicistroviridae*) open reading frames (ORF), encoding one or two long polyproteins which,

subsequently, are cleaved into functional viral proteins. Generally, these viruses undergo a covert infection pattern, but under certain environmental stresses, such as *V. destructor* infestation, they can undergo re-emergence toward an overt infection-type. Table 2 summarizes these symptoms in honeybees for the most common emerging honeybee viruses (McMenamin and Genersch, 2015).

2.2. The pathogenicity of bee virus: what we know from the honeybee

Currently, the knowledge about pathogenicity of bee virus is still limited and mainly relates to the European honeybee (*A. mellifera*) and its sister species (primarily the Asian hive bee; *A. cerana*). Mainly the pioneering work of Bailey and Ball (Bailey and Ball, 1991) during the second half of the twentieth century let to discovery and understanding of RNA viruses in honeybees. The evidence increasingly suggests a large degree of commonality of honeybee viruses among the *Apis* species (Meeus et al., 2014), usually with similar symptoms. Herein the mite, *V. destructor* plays a crucial role. Since mites directly feed on haemolymph of the honeybee, it can quickly transmit virus into host haemolymph and lead to systemic infection. In addition, uninfected mites or mites free of virus could also be easily contaminated by virus through sucking haemolymph meal from virus infected bees. These virus contaminated mites could be dangerous to uninfected hosts. Through ingestion of contaminated food, viruses could also breakthrough gut or other parts of digestion system to achieve infection. Once virus establishes systemic infection in host, most of tissues can be infected (Chen et al., 2014).

Table 2: Lists of common and emerging bee viruses

Virus	Family	*Varroa* vector status	Symptoms of overt infection in honeybees
*ABPV clade	*Dicistroviridae*	++	Paralysis; Darkened cuticle pigment; Impaired cognition and homing ability; Mortality (adult and immature bees); Colony collapse
»ALPV	*Dicistroviridae*	Unknown	Unknown
*BQCV	*Dicistroviridae*	+	Pale-yellowish, leathery cuticle of capped larva; Failure of larva to pupate; Sac-like appearance; Mortality (of larvae) ; Deceased larvae and walls of cell turn black
»BRSV	*Dicistroviridae*	Unknown	Unknown
*DWV clade	*Iflaviridae*	+++	Deformed wings; Learning deficits; Discoloring; Shortened and bloated abdomens; Mortality (adult and immature bees); Colony collapse
»LSV1/2	*Nodaviridae*	Unknown	Unknown
*SBV	*Picornavirales* (super family)	++	Pale-yellowish, leathery cuticle of capped larva; Failure of larva to pupate; Sac-like appearance; Mortality (potentially of adults, certainly capped larvae); Dead larva becomes dark, brittle scale
»TRSV	*Secoviridae*	+	Winter colony collapse? (correlative only)

This list gives a summary of the most common (designated with *) and recently emerging (designated with ») honeybee viruses, whether they are vectored by *Varroa*, and their symptoms. If *Varroa* has been shown to be a significant biological vector, the virus was designated with (+++). If the virus is frequently associated with *Varroa* but the mite has not been determined to be a biological vector, the vector status was designated with (++). Finally, if the virus is sporadically associated with tissues of the mite, or the vectoring status is in question but possible, it was designated with (+) (McMenamin and Genersch, 2015).

2.3. Bee viruses confirmed in the bumblebee

Within the intricate bee-pathogens network, infectious viruses are transmitted among different species, especially honeybees and bumblebees (Fürst et al., 2014; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010). Viruses initially reported to infect the honeybees, such as acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), deformed wing virus (DWV), black queen cell virus (BQCV), slow bee paralysis virus (SBPV)

and sacbrood virus (SBV), are also recently identified to infect bumblebees. In this project, we focused on the study of two bee viruses, IAPV and SBPV.

IAPV (Family: *Dicistroviridae*)

This virus was first isolated in honeybee colonies that suffered from severe mortality in Israel, with symptoms reminiscent of those infected with ABPV, therefore, the virus isolated was named as Israeli acute paralysis virus. The genome sequencing (Maori et al., 2007) indicated that IAPV was a distinct dicistrovirus, with single RNA genome of ~9487 nt in positive orientation, and with two open reading frames that encode viral polyproteins which are processed to functional proteins later on (Figure 2). Through transmission electron microscopy (TEM), IAPV particles in purified solutions can be visualized (Figure 3).

IAPV together with two other genetically and biologically closely related viruses, ABPV and KBV, constitute a virus complex (de Miranda et al., 2010a), which is capable of inducing rapid and acute mortality of both brood and adult honeybees, in response to unspecified environmental stressors or through active transmission by the parasitic mite *Varroa destructor*. In honeybee colonies, IAPV is established as a persistent infection, likely enabled by both horizontal and vertical transmissions (Chen et al., 2014). Through oral IAPV feeding, it can provoke neurological symptoms within 20-24 hours in honey (Galbraith et al., 2015). Effects on reproduction of bumblebees have been described after IAPV or KBV artificial feeding (Meeus et al., 2014).

Figure 2: ABPV–KBV–IAPV genomes.

The identified functional domains are the helicase, 3C-protease and the RNA-dependent RNA polymerase in the non-structural open reading frame, followed by an Internal Ribosome Entry Site (IRES) in the intergenic region, and the four capsid proteins in the structural open reading frame. An IRES is also expected in the 5′ untranslated region (5′UTR) (de Miranda et al., 2010a).

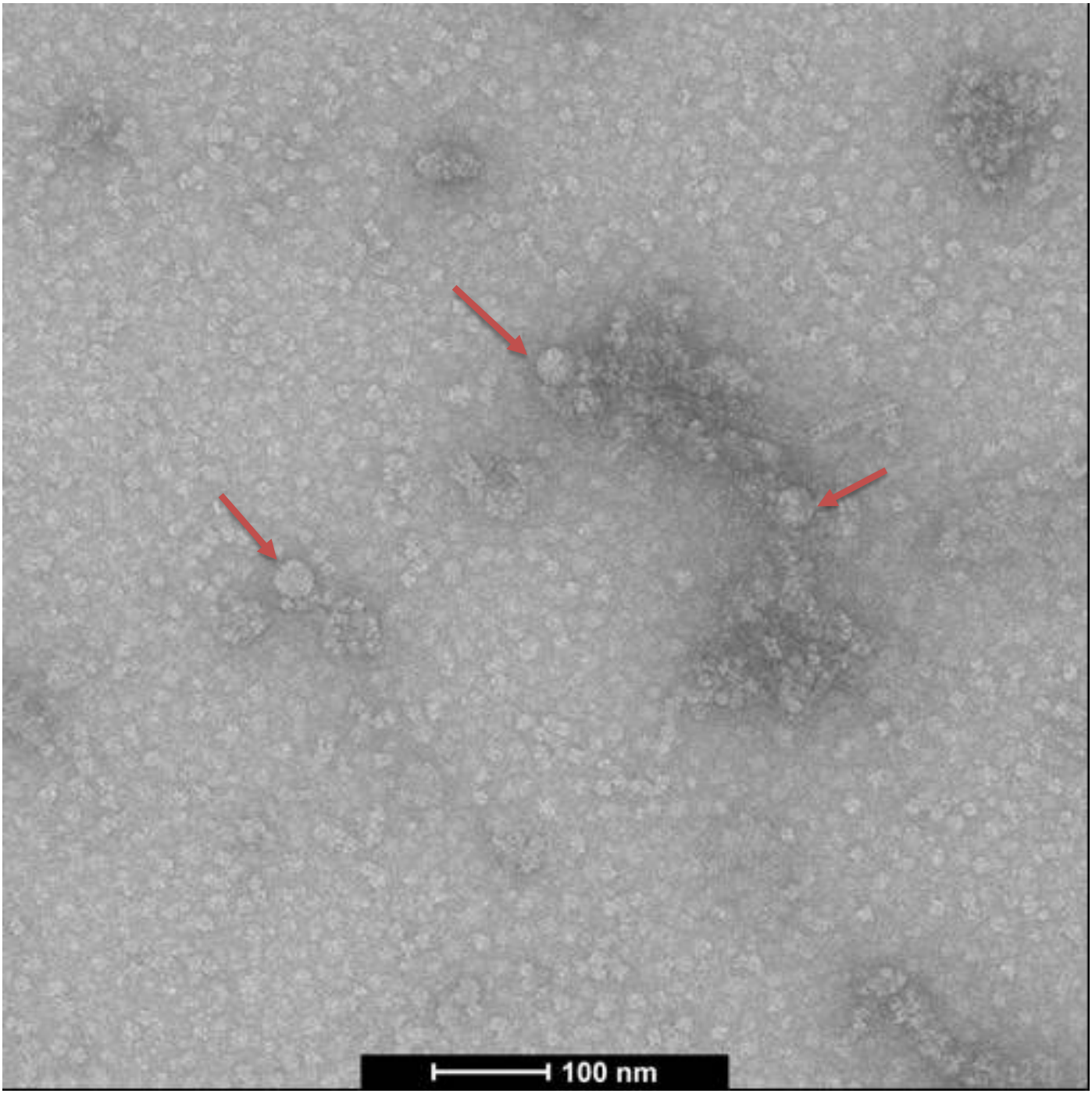


Figure 3. Representative micrograph of IAPV particles by transmission electron microscopy.

Three viral particles (arrows) with icosahedral structure and a size of approximately 34 nm. Scale bar: 100 nm.

SBPV (Family: *Iflaviridae*)

SBPV was discovered in England in 1974, it induces paralysis after injection into honeybees. Like most bee viruses, SBPV acts as a covert infection, and can be transmitted by both oral ingestion and a vector such as the *Varroa* mite. Genome (Figure 4) sequencing revealed approximately 9500 nt containing a single open reading frame (de Miranda et al., 2010b). An investigation of 847 colonies in 162 apiaries across five European countries found < 2% prevalence of SBPV (de Miranda et al., 2010b). Through TEM, SBPV particles in purified solutions can be visualized (Figure 5). A survey in the UK shows a higher prevalence of SBPV in bumblebees than in honeybees (McMahon et al., 2015).

Figure 4: SBPV genome.

The identified functional domains are the helicase, 3C-protease, the RNA-dependent RNA polymerase, and capsid proteins. (de Miranda et al., 2010b)

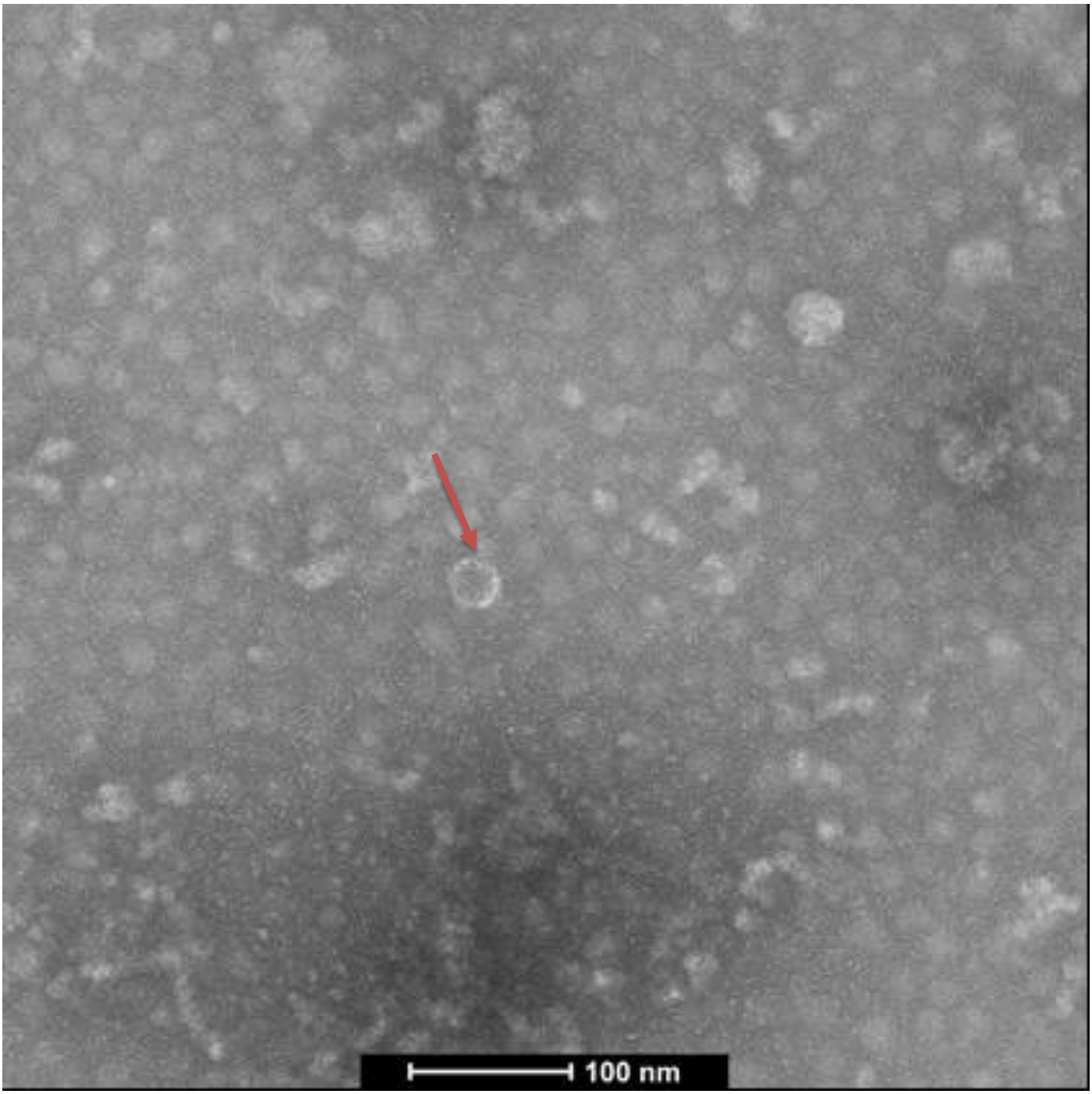


Figure 5. Representative micrograph of SBPV particles by transmission electron microscopy.

The viral particle (arrow) with icosahedral structure and a size of approximately 34 nm. Scale bar: 100 nm.

3. Bee antiviral immune system

3.1. Bee immune pathways

Being obligate intracellular parasites, the replication of a virus is dependent on the host cellular machinery. The interplay between a host and a virus leads to a constant "arms-race". In one hand the host's immune system tries to eliminate viral infections, on the other hand viruses try to surpass the host's immune system in an attempt to successfully infect the host. In addition, the host has to allocate resources to trigger immune responses during pathogen invasion, which has its trade-off against other physiological functions (Bascuñán-García et al., 2010; Hosken, 2001). The immunity response of the host is not static and often depends on viral characteristics and host physiological conditions (Schneider, 2009).

From the view of host defense, an infection occurs after a virus has breached the physical and chemical barriers, then the insects rely on their innate immunity responses, such as RNA interference (RNAi), Toll, Imd, JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) and autophagy pathways to combat viruses (reviewed by (Kingsolver et al., 2013; Merkling et al., 2013; Vijayendran et al., 2013)). Figure 6 summarizes the canonical honeybee immune pathways (Brutscher et al., 2015). The honeybee genome encodes major members of insect immune pathways including: RNAi; JAK/STAT; Toll; JNK (c-Jun N-terminal kinase); and MAPK (Mitogen-Activated Protein Kinases), as well as orthologs of genes involved in autophagy, eicosanoid biosynthesis, endocytosis, and melanization.

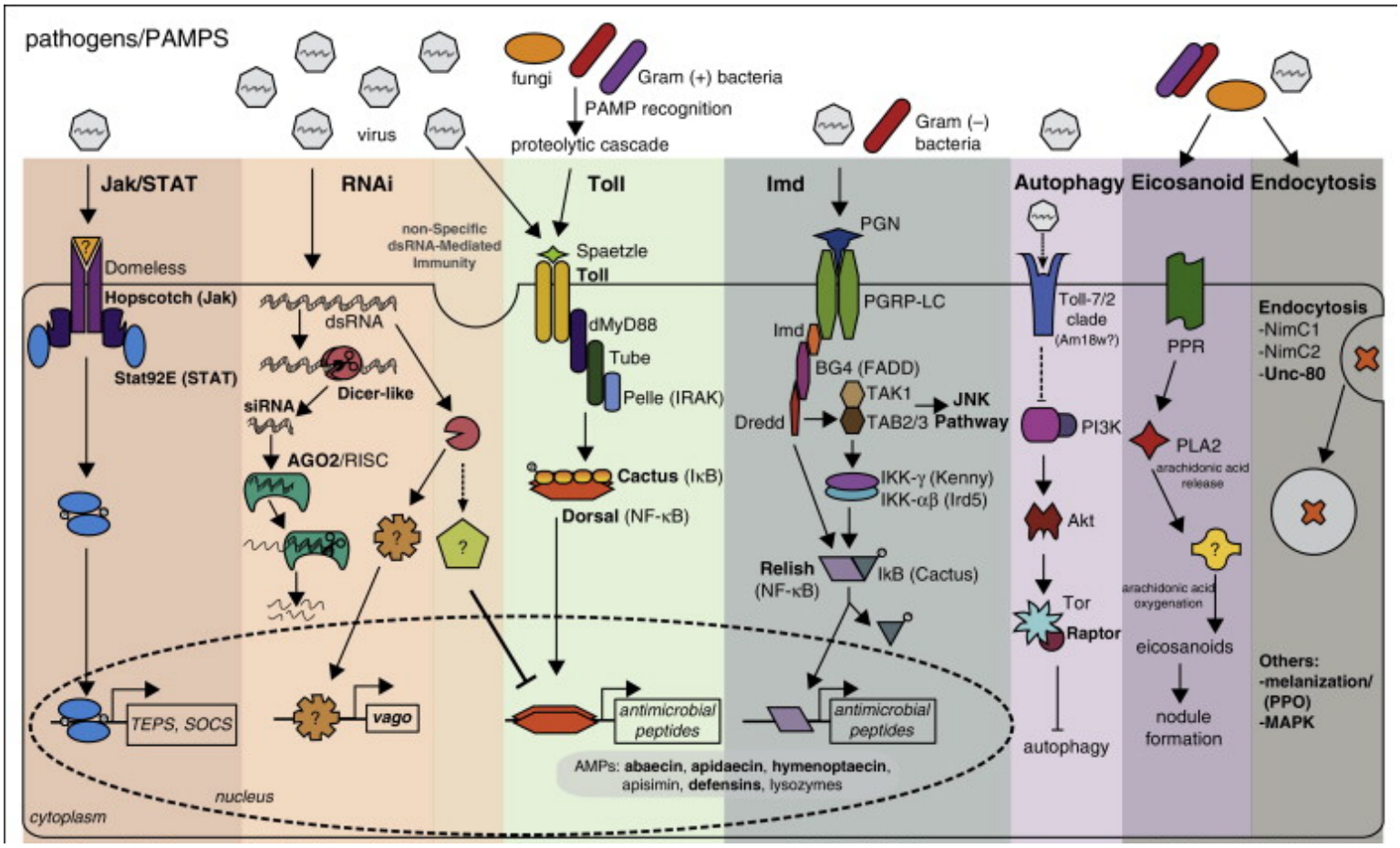


Figure 6: **Honeybee immune pathways — highlighting genes implicated in antiviral immune responses (Brutscher et al., 2015)**.

In social insects, common features like high population density and low genetic diversity are ideal for parasite transmission. One could expect that social behavior of bees plays an important role in the protection of the colonies from infection. Therefore, for bees, we need to take both individual and social immunity into consideration. Somewhat unexpected, the genes of the

immune systems of social bees and solitary bee are similar (Barribeau et al., 2015; Kapheim et al., 2015). In addition, evidence of positive selection in genes encoding antiviral responses in both social and solitary bees are also presented in Figure 7. It is known that honeybees have a relatively lower immune gene repertoire compared to other insects (Honeybee Genome Sequencing Consortium, 2006). The recent studies showing the similarity in immune complement across a gradient of sociality of bees suggests that the small set of immune repertoire is rather a bee property instead of property solely present in the social honeybee and thus predates the evolution of sociality (Barribeau et al., 2015). Based on this hypothesis, the small set of immune gene repertoire in bumblebee and honeybee compared with *Drosophila*, highlight immunity studies in bees could also enhance our understanding about immunity origin and evolution in insects.

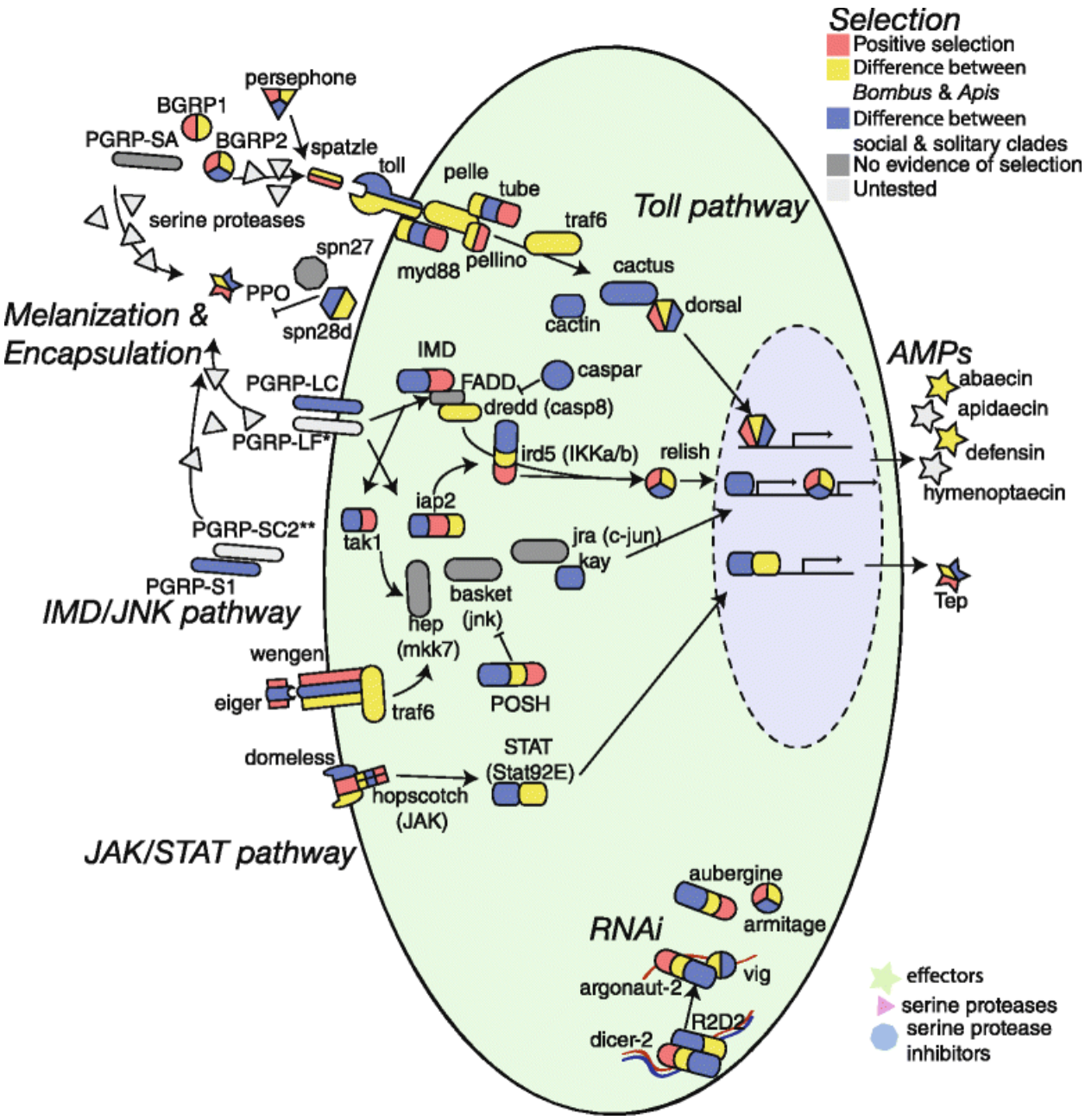
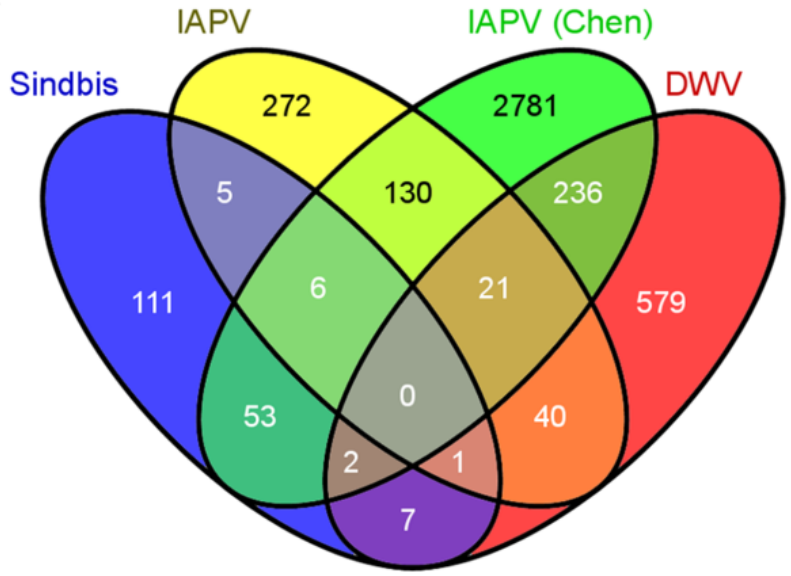


Figure 7: Diagram of the classical insect immune genes.

Colors of the genes indicate evidence of selection as detected by either positive selection (across the four taxa phylogeny, on the branch between *Bombus* and *Apis*, the branch leading to *Bombus*, *Apis*, or *Megachile*) in red, or differences in selection between *Bombus* and *Apis* (yellow), or between the social and solitary clades (blue) (Barribeau et al., 2015).

3.2. Transcriptome analysis upon bee virus infection

A venn diagram (Figure 8) (Galbraith et al., 2015) shows the overlap of transcriptional responses to bee viruses, IAPV (Chen et al., 2014; Galbraith et al., 2015), DWV (Ryabov et al., 2014) and the non-natural bee virus Sindbis virus (SINV) (Flenniken and Andino, 2013). Although a great degree of overlap for differentially expressed genes upon viral infections is revealed, the variability among different data is noticeable which could be due to differences in antiviral responses across life stages or to the different viruses (Galbraith et al., 2015).



Intriguingly, the infection of IAPV also significantly changes DNA methylation status of 156 genes in honeybee , which shows no significant overlap with the significantly differentially expressed genes (Galbraith et al., 2015).

Figure 8: Comparative analysis of transcriptional responses to different bee viruses (Galbraith et al., 2015)

Table 3 (Brutscher et al., 2015),shows that some of these differentially expressed genes upon viral infections are associated with some honeybee canonical immune pathways, such as RNAi, Toll, JAK/STAT, and Imd.

Table 3: Honeybee immune genes with differential expression in virus-infected honeybees (Brutscher et al., 2015)

Gene name	Pathway	Accession number	Virus
abaecin	AMP	NM_001011617.1	SINV
apidaecin 1 (apid 1)	AMP	NM_001011613.1	SINV,DWV
apidaecin 1 (apid73)	AMP	XM_006572699.1	SINV,DWV
apidaecin type 22 (apid22)	AMP	NM_001011642.1	SINV,DWV
hymenoptaecin	AMP	NM_001011615.1	SINV,DWV
defensin-2	AMP	NM_001011638.1	DWV
vago	antivir	XM_395092.4	DWV
nimrod c1 (nimc1)	EGF Family	XM_006561053.1	SINV
unc-80/endocytosis	Endocytosis	XM_006558847.1	SINV
dscam	IG superfamily	NM_001014991.1 XM_006567003.1– XM_006567105.1	SINV
relish (rel), var x1	IMD	XM_006562219.1	DWV
relish (rel),var x2	IMD	XM_006562220.1	DWV
relish (rel),var x3	IMD	XM_006562221.1	DWV
d-pias,var x1	JAK/STAT	XM_006561055.1	IAPV
d-pias,var x2	JAK/STAT	XM_006561056.1	IAPV
d-pias,var x3	JAK/STAT	XM_623568.4	IAPV
hopscotch (hop),var x1	JAK/STAT	XM_001121783.3	IAPV
hopscotch (hop),var x2	JAK/STAT	XM_006567688.1	IAPV
hopscotch (hop),var x3	JAK/STAT	XM_006567689.1	IAPV
hopscotch (hop),var x4	JAK/STAT	XM_006567690.1	IAPV
stat92e-like	JAK/STAT	XM_397181.5	IAPV
Raptor	PI3K-Akt-Tor	XM_624057.4	IAPV
argonaute 2 (ago2)	RNAi	XM_395048.5	IAPV
dicer-like	RNAi	XM_006571316.1	IAPV
nf-κ-β inhibitor cactus 1	Toll/TLR	NM_001163712.1	IAPV
toll-6	Toll/TLR	XM_393712.4	IAPV
dorsal,var a	Toll/TLR	NM_001011577.1	IAPV

3.3. The immune response of the siRNA pathway in the defense against bee viruses

RNAi is activated by double-stranded RNA (dsRNA) which leads to the down-regulation of gene expression at the post-transcriptional level. The RNAi mechanism can be divided into three major pathways based on the type of the small RNAs produced: microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (Vijayendran et al., 2013; Wilson and Doudna, 2013). In comparison with other insects, bumblebee genomes encode common components of RNAi pathways (Table 4). During viral infection, the siRNA pathway is triggered by virus-derived dsRNAs, which finally results in cleavage of viral RNA. Here we focus on the current research progress in the understanding of the siRNA pathway of bees, its response during viral infection, and its applications in the protection of pollinator health.

The molecular mechanism of the siRNA pathway and its antiviral action

During viral infection, virus-related dsRNAs are generated, such as replication intermediates, viral genome itself with dsRNA structure, virus-encoded siRNAs and viral transcript-genome hybrids (Marques et al., 2013; Vijayendran et al., 2013). Those virus-related dsRNAs are recognized by the host and processed into 21 to 22 nucleotide-long vsiRNAs by a ribonuclease III (RNase III) enzyme called *Dicer-2*; then the vsiRNAs are loaded onto Argonaute (*Ago-2*), forming the RNA-induced silencing complex (RISC). Then the passenger strand of the vsiRNAs is degraded and the other strand (guide strand) serves as a viral sequence-specific guide to degrade viral RNA by complementary binding (Figure 9).

Table 4: Overview of the genes coding for RNAi core machinery proteins in various insect species (Sadd et al., 2015).

Gene family	*D. melanogaster*	*T. castaneum*	*B. mori*	*A. mellifera*	*B. terrestris*	*B. impatiens*
RNAse III	Dicer-1	Dicer-1	Dicer-1	Dicer-1	Dicer-1	Dicer-1
	Dicer-2	Dicer-2	Dicer-2	Dicer-2	Dicer-2	Dicer-2
	drosha	drosha	drosha	drosha	drosha	drosha
Argonaute	Ago-1	Ago-1	Ago-1	Ago-1	Ago-1	Ago-1
	Ago-2	Ago-2	Ago-2	Ago-2	Ago-2	Ago-2
	Ago-3	Ago-3	Ago-3	Ago-3	Ago-3	Ago-3
	aubergine	aubergine	aubergine	aubergine	aubergine	aubergine
	piwi	piwi				
Genes coding	pasha	pasha	pasha	pasha	pasha	pasha
dsRNA binding	R2D2	R2D2	R2D2	R2D2	R2D2	R2D2
proteins	loquacious	loquacious	loquacious	loquacious	loquacious	loquacious

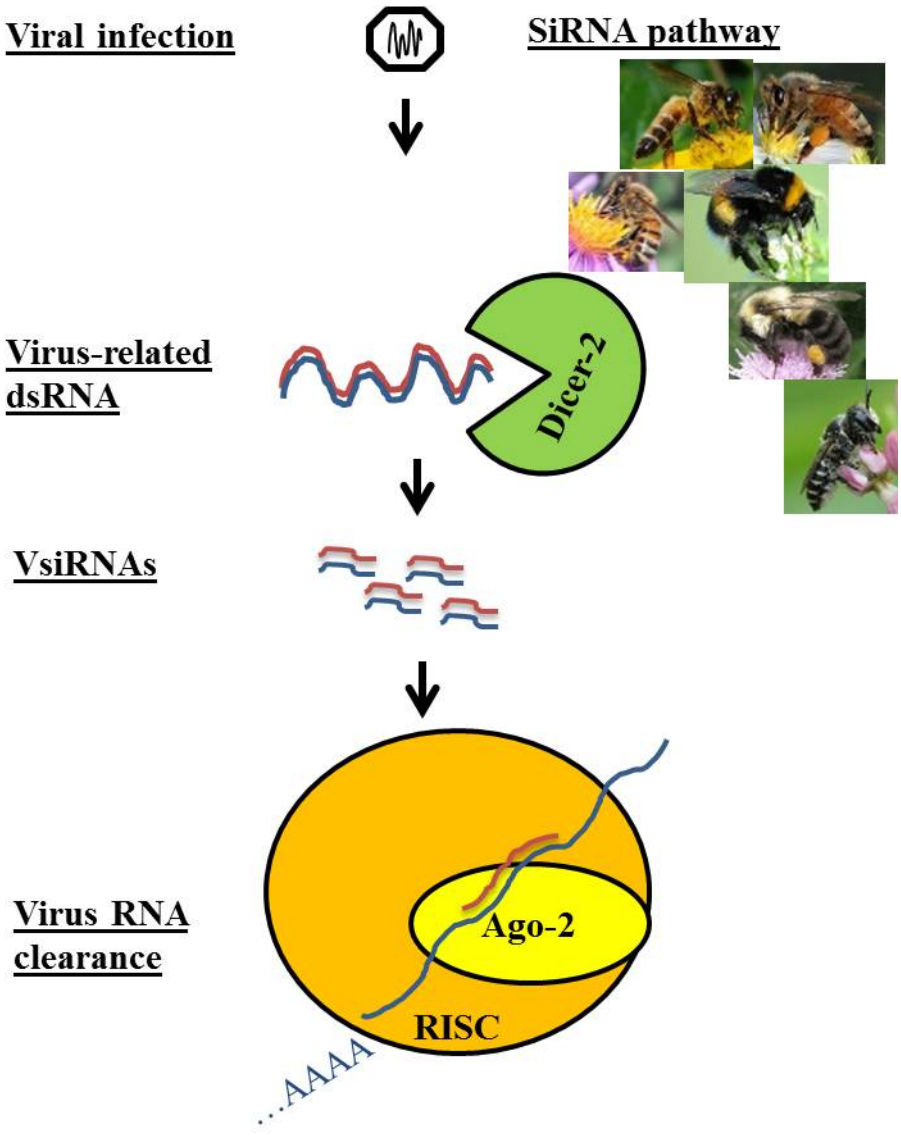


Figure 9: Proposed response of the siRNA pathway to viral infection in bees.

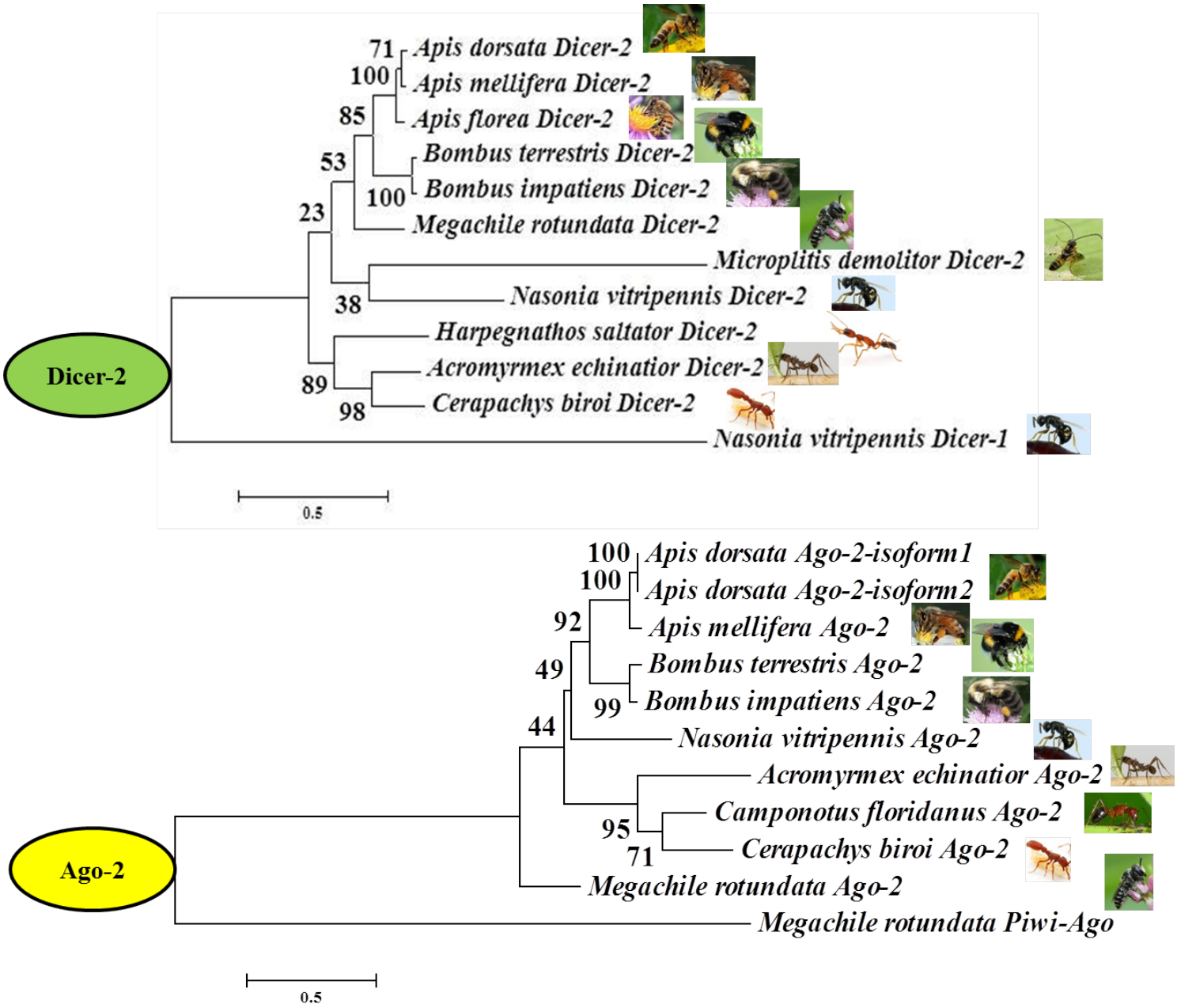


Figure 10: Phylogenetic trees of *Dicer-2* and *Ago-2* (Niu et al., 2014b).

Phylogenetic trees were constructed by MEGA 6.0 (Tamura et al., 2013). The sequences from *Bombus terrestris* (XP_003394821.1), *B. impatiens* (XP_003485689.1), *Megachile rotundata* (XP_003703800.1), *Harpegnathos saltator* (EFN79336.1), *Apis florea* (XP_003697097.1), *A. dorsata* (XP_006623214.1), *A. mellifera* (XP_006571379.1), *Acromyrmex echinatior* (EGI69620.1), *Microplitis demolitor* (EZA46212.1), *Cerapachys biroi* (EZA61552.1), *Nasonia vitripennis* (XP_001605287.1, XP_001602524.2) were used for *Dicer-2* based on RNase III and PAZ domains; the sequences from *B. terrestris* (XP_003398529.1), *B. impatiens* (XP_003492410.1), *A. mellifera* (XP_395048.4), *A. dorsata* (XP_006625010.1), *A. dorsata* (XP_006625011.1), *M. rotundata* (XP_003705687.1, XP_003708345.1), *N. vitripennis* (XP_008214884.1), *A. echinatior* (EGI64275.1), *Camponotus floridanus* (EFN68927.1), *C. biroi* (EZA61145.1) were used for Ago-2 based on PAZ and Piwi domains.

To achieve an effective antiviral activity by the siRNA pathway it is also crucial to pass on the local siRNA response in infected cells to uninfected cells. This mechanism is called a systemic response of RNAi, although the exact signaling molecules are unknown, it normally requires uptake of dsRNA by uninfected cells (Saleh et al., 2009). This process could be important for viral immunity as insects, unlike vertebrates, lack an adaptive immune system. The uptake of virus-related dsRNA by uninfected cells could prime these cells for an effective immune response upon viral infection. Currently, two dsRNA uptake mechanisms are described in insects, transmembrane channel-mediated uptake and endocytosis-mediated uptake (Huvenne and Smagghe, 2010; Scott et al., 2013). Little is known about dsRNA uptake or the spreading of RNAi signals in bees, but it seems that honeybees are inefficient in spreading RNAi signals such as siRNAs across tissues (Jarosch and Moritz, 2011). Moreover, in most cases the silencing of genes in honeybees or bumblebees requires high amounts of dsRNA, especially compared with that in the desert locust *Schistocerca gregaria* (Wynant et al., 2012). The latter may suggest a low dsRNA uptake efficiency in bees. *SID-1*, a multispan transmembrane protein, is speculated to be an important factor in systemic RNAi, however, its function in bees is still unknown. While it should be remarked that after silencing the honeybee Toll-related receptor 18W, the expression level of the transmembrane protein *SID-1* increased 3.4-fold, while the target gene's expression was decreased 60-fold (Aronstein et al., 2006). The latter may indicate a role of *SID-1* in dsRNA uptake, but solid evidence is still lacking. Therefore, two questions concerning dsRNA uptake in bees: i) What is the mechanism for dsRNA uptake? and ii) What is the contribution of virus-related dsRNA uptake in controlling viral infection? need to be addressed in future studies.

Silencing genes in honeybees and bumblebees has been achieved by administrating gene-specific

dsRNA, and titers of honeybee viruses can also be reduced with virus-specific dsRNA (including siRNA) (Chen et al., 2014; Desai et al., 2012; Maori et al., 2009). These studies confirmed the conserved function of the siRNA pathway. To better understand the molecular aspects of this pathway, we searched for available protein sequences of core components of the siRNA pathway such as *Dicer-2* and *Ago-2* in bees, ants, and wasps from Genbank, analyzed the predicted domains, and then phylogenetic trees were constructed (Figure 10). Analyzed by HMMER (http://hmmer.janelia.org/), RNase III and PAZ domains in *Dicer-2*-like proteins, PAZ and Piwi domains in *Ago-2*-like proteins were found in bees, ants and wasps. All the sequences of *Dicer-2* and *Ago-2* were clearly separated from their close counterparts *Dicer-1* (*Nasonia vitripennis*), and *Piwi-Ago* (*Megachile rotundata*), respectively (Figure 10). It was not surprising that the sequences were clustered together based on taxonomic kinship. Insect behavior (social vs. solitary lifestyle) seemed to have no influence on the similarity of the genes. This seems to be consistent with the genome level comparison of bees from social to solitary which shows immune genes may be predated before evolution of sociality (Barribeau et al., 2015). To prove the relation of these genes with the siRNA response, further study is required, and here the RNAi-of-RNAi approach is proposed as a useful technique to evaluate the involvement of these core proteins in insects (Wynant et al., 2012). This approach is widely used to deplete the core gene expression of RNAi pathway in order to study its function.

Responses of the siRNA pathway upon viral infection

Deep-sequencing analysis of samples collected from colonies suffering from colony collapse disorder revealed abundant siRNAs of 21-22 nucleotides perfectly matching the IAPV, KBV and DWV genomes (Chejanovsky et al., 2014). To further confirm if these small RNAs were derived from viruses, honeybees experimentally infected with IAPV showed a high incidence of small

RNAs matching the IAPV genome (Chejanovsky et al., 2014). In addition, small RNAs matching to *Varroa destructor* virus-1 (VDV-1) and DWV genomic sequences were also found in field-collected honeybees but not in bumblebees (Wang et al., 2013a). Lack of detection of these RNAs in these bumblebees without virus pre-screening could be caused by limited sample collection, as DWV and VDV-1 can infect bumblebees and other pollinators (Fürst et al., 2014; Levitt et al., 2013; Singh et al., 2010). Therefore, it can be concluded that the siRNA pathway in bees can generate virus-derived small interfering RNAs (vsiRNAs) from various viruses. The siRNA response in multi-virus infections is still unclear since these two studies used pooled samples for sequencing and the infection status of the individual bees was not confirmed. DWV, when transmitted by *V. destructor* mites, can directly be delivered into the hemolymph of honeybees, thereby giving DWV an advantage over its host, facilitating replication and spread, which can lead to high virus titers (Martin et al., 2012). Although the significant changes in expression of *Dicer-2* and *Ago-2*, were absent in bees, vsiRNAs matching to DWV were detected by small RNA sequencing. Moreover, the intensity of infection seemed to be correlated with the amount of vsiRNAs, indicating that the siRNA response is proportional to the intensity of the viral infection (Ryabov et al., 2014). However, one needs to consider that these high levels of vsiRNAs do not necessarily mean an RNAi antiviral action. Indeed virus encoded suppressors of RNAi (VSR) may inhibit downstream activity of RNAi, for instance, inhibiting slicer activity of *Ago-2* (van Mierlo et al., 2012). Recently, it has been suggested that IAPV encodes a VSR (Chen et al., 2014). However, the data is not yet conclusive and needs further analysis.

Beyond the siRNA pathway, also Toll, Imd and JAK/STAT pathways may be activated during viral infection (Merkling et al., 2013). Intriguingly, the siRNA and the JAK/STAT pathways perform cross-talk in mosquitoes in a *Dicer-2*-dependent manner through the action of a secreted

signaling molecule, namely *Vago*, leading to an antiviral defense state in uninfected cells (Kingsolver and Hardy, 2012; Paradkar et al., 2012). Apart from virus-specific dsRNA generated during viral infections, non-specific dsRNA also seems to mediate an antiviral response in reducing viral titers. Co-injection of non-specific dsRNA with a model virus, the recombinant Sindbis virus with green fluorescent protein (SINV-GFP), to honeybees, showed reduced SINV-GFP titers (Flenniken and Andino, 2013). Both non-specific dsRNA and SINV-GFP significantly decreased the expression of various Antimicrobial peptides (AMPs) in these bees, but the majority of genes for which the transcription levels increased were not canonical insect immune genes. In bumblebees, non-specific dsRNA and IAPV specific dsRNA showed a relatively similar level of antiviral effects to IAPV (Piot et al., 2015). So far, three hypotheses can be drawn about the involvement of a non-specific dsRNA in the induction of the immune response: i) non-specific dsRNA induces the siRNA immune response to some extent, and this helps to restrict viral infection; ii) dsRNA can work as a pathogen associated molecular pattern (PAMP) which are recognized by the host thus triggering other immune pathways; iii) Non-specific dsRNA is recognized by different immune pathways, including the siRNA and some other immune pathways, the antiviral response is a combined effect from various pathways.

Using the siRNA pathway to control bee viruses

Through ingestion of IAPV-specific dsRNA or siRNA in honeybees infected with IAPV, the IAPV titers were reduced (Chen et al., 2014; Maori et al., 2009). Feeding larvae with DWV-specific dsRNA before inoculation with DWV reduced the DWV viral titer and wing deformity, while feeding adult workers with DWV-specific dsRNA in advance of inoculation with DWV increased their longevity and reduced DWV titers. In addition, direct feeding DWV-specific dsRNA did not affect larval survival rates which suggests that it is non-toxic to larvae (Desai et

al., 2012). Also ingestion of SBV-specific dsRNA could significantly reduce virus titers in SBV-infected bees (Liu et al., 2010). Besides laboratory conditions, the large-scale field application of this strategy is also able to reduce the IAPV disease in honeybees. These studies together demonstrate the use of targeted treatments for viral diseases in bees by using the innate RNAi immune pathway (Hunter et al., 2010). Moreover, dsRNA ingested by bees can be transferred to the *Varroa* mite and from the mite onwards to a parasitized bee. This bidirectional transfer of dsRNA between honeybee and *V. destructor* can lead to an approach to use RNAi to control mites, thereby reducing virus transmission (Garbian et al., 2012).

In conclusion, during viral infection, the siRNA pathway in bees is activated and thus leads to the degradation of the viral RNA or its genome, therefore playing a major role in the defense against different viruses in bees. Moreover, the bees can be protected through the introduction of virus specific-dsRNA in large scale field applications. However, there are still some questions that need to be addressed in the future: i) What is the involvement of the siRNA pathway in multi-virus infections? ii) What is the influence of pre-infection with a non-virulent virus (or persistent infection) on the siRNA pathway, and subsequent effect to the infection of other viruses? iii) What kind of factors can enhance the activity of siRNA pathway? iv) How does the host sustain the balance between its siRNA immune investment to control virus and other stressors presented, such as food shortage, pesticides, parasite mites or other pathogen load.

3.4. The miRNA pathway

MicroRNAs (miRNA) play an important role in regulating gene expression and influence various biological processes in eukaryotes. In insects, the miRNA pathway has been documented to be involved in different aspects of development, such as formation of germ cells, wing, and muscle, neurogenesis, apoptosis, and phenotypic plasticity (Asgari, 2013). The miRNA pathway is also

well established to be involved in host-pathogen interactions (Asgari, 2013; Hussain and Asgari, 2014b). The canonical biogenesis (Figure 11) of miRNA initiates in the nucleus where monocistronic, bicistronic or polycistronic transcripts are produced. These contain stem-loop structures known as the primary miRNA (pri-miRNA). The pri-miRNA is cleaved by Drosha and Pasha to liberate the precursor miRNA (pre-miRNA). After exportation to the cytoplasm, the pre-miRNA is cut by Dicer-1 to yield a miRNA duplex. The duplex strands are then sorted and the miRNA strand is loaded into RNA induced silencing complex (RISC) which typically includes Argonaut 1 (Ago-1). Then, the mature miRNA binds to the target mRNA and leads to mRNA degradation or translational repression (Lucas and Raikhel, 2013).

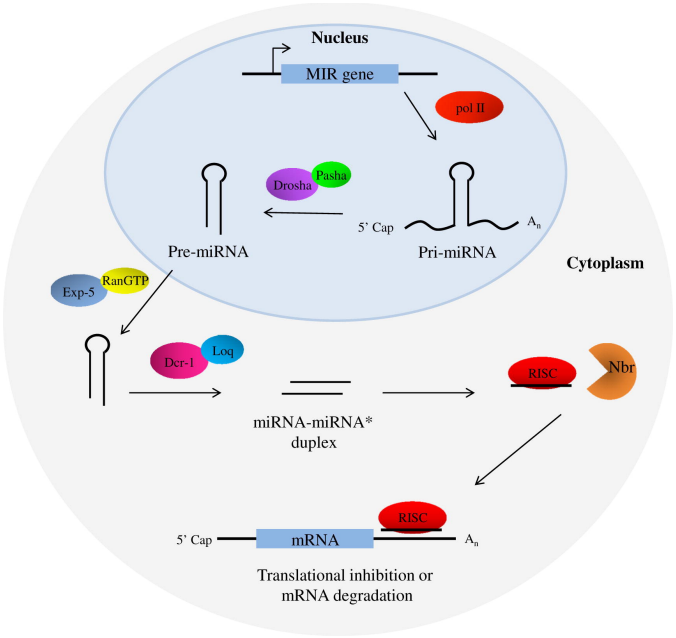


Figure 11. A model for microRNA biogenesis (Lucas and Raikhel, 2013).

Besides, there are also non-canonical pathways of miRNA biogenesis, which are Drosha-independent but can be Dicer-dependent or Dicer-independent (Yang and Lai, 2011). The production and regulatory effects of miRNAs on insect-virus interactions could be complex. The first layer of complexity relates to the origin of miRNAs, which could be from the host or the virus. The second layer of complexity arises from the two-way interplay, meaning host encoded miRNAs can target genes from both host and viral genes and *vice versa* for virus encoded miRNAs (Figure 12) (Asgari, 2015).

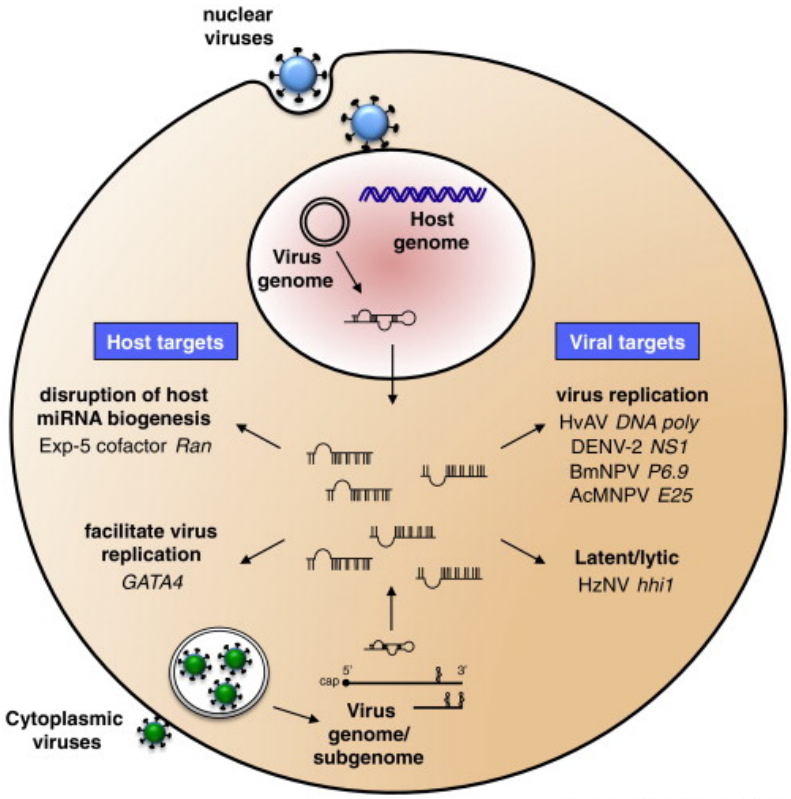


Figure 12: Virus-encoded miRNAs could be produced by nuclear or cytoplasmic viruses through canonical or non-canonical pathways (Asgari, 2015).

Differential expression of miRNAs has been associated with honeybee development and social behavior (Behura and Whitfield, 2010; Greenberg et al., 2012; Liu et al., 2012b; Weaver et al., 2007). Recently, with the genome sequencing of two bumblebee species, *Bombus terrestris*, and *B. impatiens*, two datasets of miRNAs have been annotated for both species (Sadd et al., 2015). In comparison of miRNAs between the bumblebees and honeybees (Figure 13), unique miRNAs are identified in both groups. A total of 116 miRNAs were found in the genomes of all three species. Strikingly, 103 miRNAs of the 219 in *A. mellifera* were not found in the genome of either bumblebee species. These unique miRNAs are excellent candidates that may tune key biological differences between advanced eusocial honeybees and primitively eusocial bumblebees (Sadd et al., 2015).

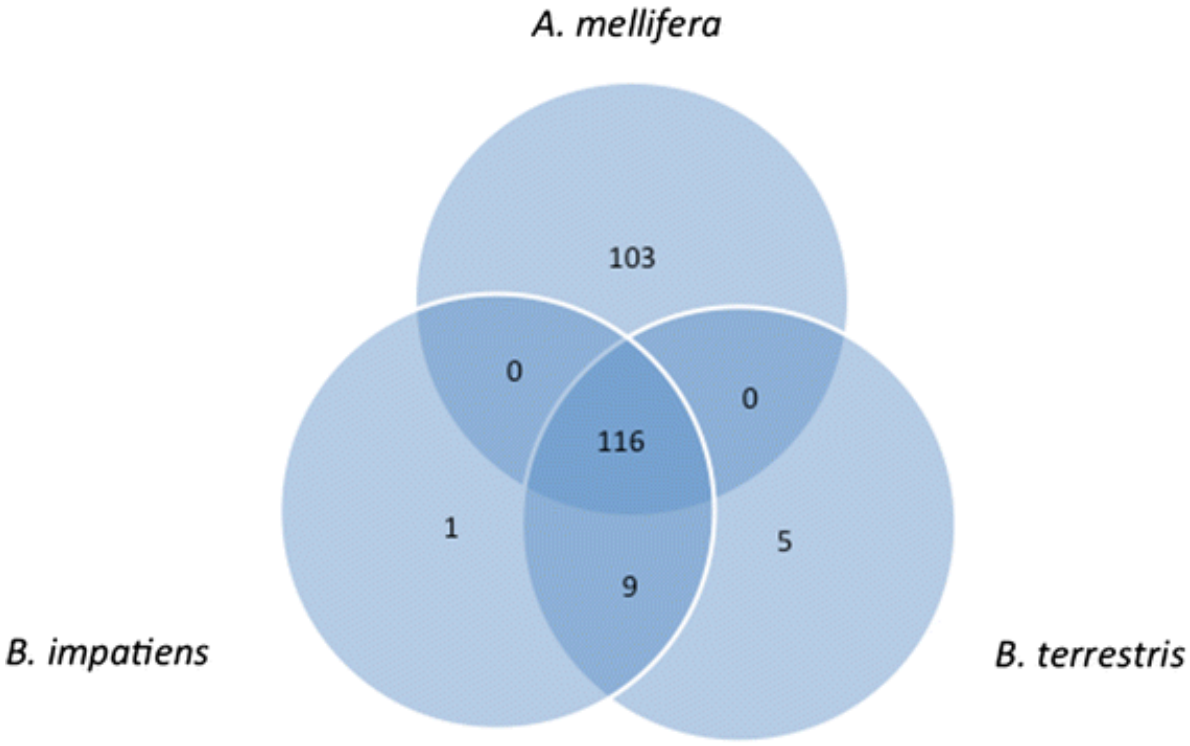


Figure 13: Venn diagram of the distribution of unique and shared miRNAs across the two bumblebee species investigated and *Apis mellifera* (Sadd et al., 2015).

3.5. The JAK/STAT pathway

Upon virus invasion or certain stresses, the JAK/STAT pathway is generally initiated by the ligand unpaired (Upd) binding to the transmembrane receptor Domeless (Dome) which is a distant homolog of the vertebrate type I cytokine receptor. However, the homologues of Upd in bees is not identified (Figure 14). The conformational change of Dome after Upd binding leads to the self-phosphorylation of the Janus kinase Hopscotch (Hop).

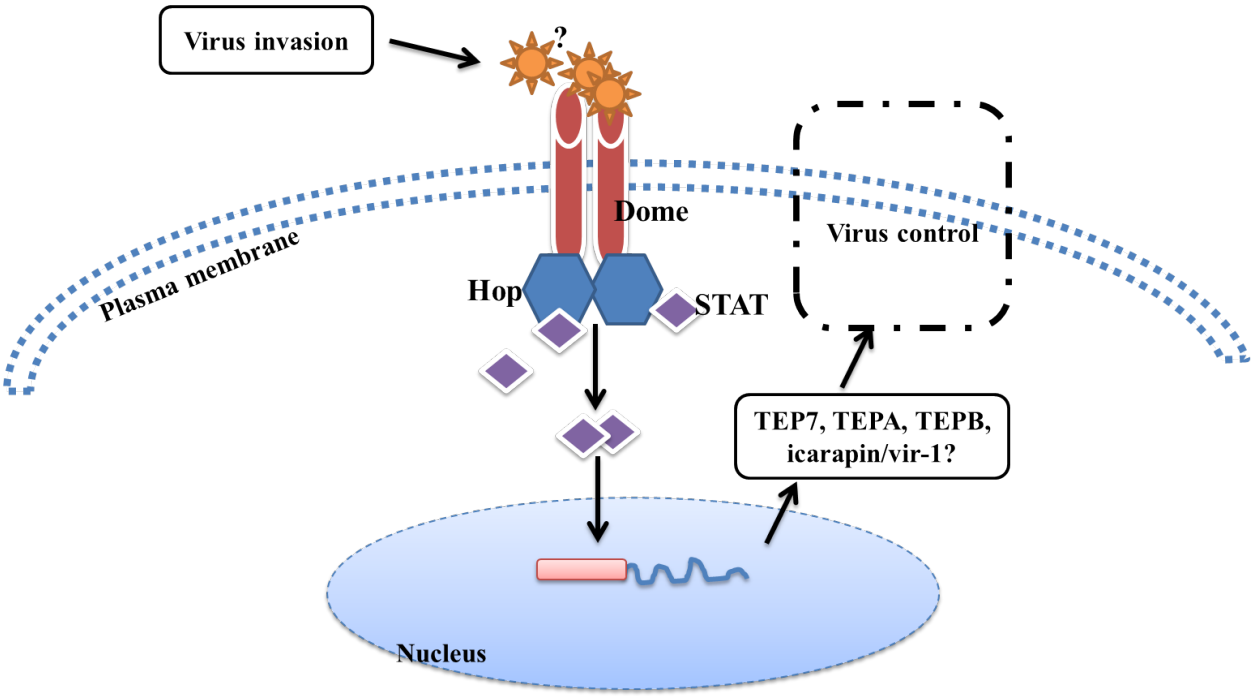


Figure 14: Proposed JAK/STAT pathway upon viral invasion in bees.

The activated Hop will phosphorylate Dome, thereby forming docking sites for the cytoplasmic signal transducer and activator of transcription (STAT). The recruitment of STAT to these docking sites enables Hop to phosphorylate STAT which leads to its dimerization. Subsequently, the STAT dimers translocate to the nucleus where they activate transcription of specific effector

genes to achieve controlling of viruses (Myllymäki and Rämet, 2014). Activated STATs transcriptionally regulate antimicrobial effectors TEP7 (thioester-containing protein 7), TEPA and TEPB. Virus induced gene 1 (*vir-1*) is the downstream transcribed gene of JAK/STAT during virus infection in *Drosophila* (Deddouche et al., 2008; Dostert et al., 2005). However, it is less clear whether this protein is presented in bees. The protein sequence of *vir-1* in *Drosophila* and mosquito showed a significant similarity to a newly identified protein in honeybees, namely icarapin, a novel IgE-binging venom protein, which can evoke an immune response in subjects after a bee sting (Peiren et al., 2006).

Chapter II-*In vivo* study of *Dicer-2* mediated immune response from the small interfering RNA pathway upon viral infections in bumblebees (*Bombus terrestris*)

Parts of this chapter were submitted to:

Niu J, Smagghe G, Coninck DID, Nieuwerburgh FV, Deforce D, Meeus I. (2015) In vivo study of *Dicer-2* mediated immune response from the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in *Bombus terrestris*. Insect Biochemistry and Molecular Biology Submitted.

Parts of this chapter were published in:

Niu J, Cappelle K, de Miranda JR, Smagghe G,Meeus I: Analysis of reference gene stability after Israeli acute paralysis virus infection in bumblebees *Bombus terrestris*. *Journal of Invertebrate Pathology.* 2014, 115:76-79.

1. Introduction

Bee pollination is an indispensable component of global food production and plays a crucial role in sustainable agriculture. The recent decline of bee colonies including wild populations poses a threat to this system (Goulson et al., 2015; Potts et al., 2010). Bees share common food sources and habitats, thereby form an intricate host-pathogen network where infectious diseases can be transmitted among different species, especially honeybees and bumblebees. In this network, viruses represent one of the most important groups of pathogens. (Fürst et al., 2014; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010). The common bee viruses are from the families *Dicistroviridae* and *Iflaviridae*. These viruses are non-enveloped small icosahedral virions, containing a positive sense single stranded RNA genome. Their genomes are around ~9 kb and contain one (in case of the *Iflaviridae*) or two (in case of the *Dicistroviridae*) open reading frames, encoding one or two long polyproteins which, subsequently, are cleaved into functional viral proteins. Viruses initially reported to infect the honeybees, such as acute bee paralysis virus, IAPV, KBV, DWV, BQCV, SBPV and sacbrood virus (SBV), are also recently identified to infect bumblebees (Fürst et al., 2014; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010). Effects on reproduction of bumblebees have been described after IAPV or KBV artificial feeding (Meeus et al., 2014). In honeybees IAPV is established as a persistent infection in colonies and the link with winter mortality has been reported (Chen et al., 2014). In honeybees, parasite mites, such as *Varroa destructor*, act as a vector for virus transmission (Martin et al., 2012; Ryabov et al., 2014). Tracheal mites, such as *Locustacarus buchneri*, infest on ~30 species

of bumblebees (Goka et al., 2006; Rożej et al., 2012; Yoneda et al., 2008), while it is still unclear whether these mites could play a role in virus transmission. Commercial colonies including honeybees and bumblebees are widely transported around the world, which could disrupt local host-virus interactions in sympatric wild bees. Indeed, pathogen spillovers between managed colonies after transportation and wild bees is a risk, particularly, for endangered bee populations (see review (Meeus et al., 2011)).

RNAi can be divided into three pathways based on different types of the small RNAs produced, including: miRNAs, siRNAs and Piwi-interacting RNAs. For the siRNA pathway, briefly, long dsRNA is processed into small RNAs by Dicer; these small RNAs are then loaded onto Ago, forming the RISC with other proteins (Wilson and Doudna, 2013). The guide strand of siRNAs serves as a sequence-specific guide to cleave target mRNA by complementary binding. During viral infection, especially for RNA viruses, virus related dsRNAs are generated, such as, viral dsRNA replication intermediates, the viral genome itself, and viral transcripts (Marques et al., 2013). These virus related dsRNAs can trigger an antiviral response of the siRNA pathway (Wang et al., 2015). Currently, research has been focused on two aspects of the siRNA pathway mediated antiviral response in bees (see review (Niu et al., 2014b)): 1) deep-sequencing analysis revealed several bee vsiRNAs in honeybees (Chejanovsky et al., 2014; Ryabov et al., 2014; Wang et al., 2013a); 2) the utilization of the siRNA pathway to restrict viral replication through viral sequence specific dsRNAs (Chen et al., 2014; Desai et al., 2012; Liu et al., 2010; Maori et al., 2009). Intriguingly, non-specific dsRNA also seems to mediate an antiviral response by restricting viral replication through an unknown mechanism (Flenniken and Andino, 2013; Piot et al., 2015). These studies provide some basic understanding of the interaction between the siRNA pathway and bee virus, but the antiviral response of the siRNA pathway is still poorly

studied during the systemic infection of virulent and avirulent viruses. Recently, genome sequencing of two key bumblebee species, *Bombus terrestris* and *B. impatiens* (Sadd et al., 2015) and the evolution analysis of immune genes in bees (Barribeau et al., 2015), highlights the importance to study the immune responses in primary social insect in order to better understand the evolution of insect antiviral immunity. Therefore, in this study, we comparatively analyzed the siRNA antiviral response after inducing systemic infections of IAPV and SBPV in bumblebees based on three approaches: 1) analysis of core gene expressions of the siRNA upon virus infection; 2) deep sequencing to analyze viral derived small RNAs in virus infected samples; 3) using "RNAi of RNAi" to check the virus infection after silencing one of the core genes of the siRNA pathway, *Dicer-2*. Our results provide a comprehensive insight in the involvement of bumblebee siRNA pathway, especially, characterizations of the antiviral activities possibly mediated by Dicer-2 in the control of different viruses.

2. Materials and methods

2.1. Insects and viruses

Newly emerged workers were collected from the colonies of *B. terrestris* obtained from Biobest NV (Westerlo, Belgium), and kept in micro-colonies fed with pollen and sugar water *ad libitum* for further experiments. All the micro-colonies were maintained in an incubator (Panasonic, Japan) at 29-31°C, 60-65% relative humidity, and continuous darkness. IAPV and SBPV inocula were produced in PBS by following the protocol as described previously (Niu et al., 2014a). The virus particles were counted by using transmission electron microscopy. Bees used in this study were screened by RT-PCR (primers in supplementary table S1) to make sure that they were free

of IAPV and SBPV.

2.2. Infections and survival analysis

To test the mortality caused by viral injection, serial dilutions were made and injected in five to eight day old workers by the nanoinjector (Eppendorf, Germany). The viral solution was injected in the side of soft white-like cuticle between the 1st and 2nd abdominal segments. The amount of viruses, IAPV with a range of 20 to 2,000 particles, and SBPV with a range of 2,000 to 200,000 particles, was ascertained by a pre-experiment. The mortality was checked each day until all bees died for IAPV treatment. Most bees survived after injection of SBPV, and the survival rate was followed for at least six weeks after injection of SBPV. The PBS used to make virus inocula, was applied to inject bumblebees as a control. We used 15~20 replicates for each dilution injection. The survival curves were analyzed by Kaplan-Meier in SPSS statistics 22.

2.3. Analysis of reference gene stability in bumblebee

Firstly, we selected more than 10 candidates reference genes in bumblebee due to their homologies showed stable expressions in other virus-host interaction experiments (Jorgensen et al., 2006; Liu et al., 2012a; Maroniche et al., 2011). With pre-evaluation, such as, primer amplification efficiency and melting curves, we eventually chose five candidate reference genes (supplementary data Table S1) from different gene families, namely Elongation factor-1 α (*ELF1α*), peptidylprolyl isomerase A (*PPIA*), 60S ribosomal protein L23 (*RPL23*), TATA-binding protein (*TBP*) and polyubiquitin (*UBI*), for their stability analysis. Subsequently, these five candidate reference genes were evaluated in two series of *B. terrestris* samples. The 1st series consisted of separate extracts of whole bumblebee bodies and isolated bumblebee body parts (head, gut and remnants: the remaining part of bumblebee separated from head and gut).

For the 1st series of experiments, we collected five to eight days-old workers, from which four whole body samples were prepared and three samples of each body part (head, gut and remnants; separated under a binocular microscope). For the 2nd series of experiments, we produced an IAPV inoculum by propagating an IAPV reference isolate (Allen and Ball, 1995) in 50 white-eyed honeybee pupae and preparing a chloroform-clarified extract in PBS (de Miranda et al., 2013). This IAPV inoculum had <0.1% contamination with other common honeybee viruses, as determined by RT-qPCR using previously published assays for IAPV, ABPV, KBV, Chronic bee paralysis virus, DWV, *Varroa destructor* virus-1 (VDV-1), SBPV, SBV and BQCV (Locke et al., 2012). The identity of the reference isolate and propagated inoculum was confirmed by sequencing the IAPV PCR product. Five to eight day-old workers were injected with 2 µl IAPV inoculation solution (20 IAPV particles), or 2 µl PBS. Afterwards, four individuals were collected for the IAPV-injected or control samples at each time moment of 8, 24, 48 and 72 h.

The stability of the different reference genes were calculated by GeNorm^{PLUS} (Hellemans et al., 2007). Although different default limits of *M* values have been used in various studies as ≤1.5 (Zhang et al., 2012), ≤1.0 (Shen et al., 2010), and ≤0.5 (Everaert et al., 2011), we have chosen in our study the strict *M* value of 0.5 as considering the number of candidate reference genes studied and the number of samples to validate the candidate reference genes. The stability ranking of the candidate reference genes was also obtained by NormFinder, in contrast to GeNorm, it also accounts for optimum reference gene out of a group of genes from diverse samples (Andersen et al., 2004) e.g. from individual body parts, whole bodies, virus infected and non-infected bumblebees in our case.

2.4. Dynamics of genome copy number during virus infections

To screen viral genome copy number (gcn) during infection, we collected samples at 8 h, 1, 2, and 3 days post injection (dpi) for IAPV (20 particles per bee for injection) and 8 h, 1, 2, 3, 7 and 13 dpi for SBPV (20,000 particles per bee for injection). For each biological replicate, the whole body of each individual bee was used to extract RNA. Relative viral gcn was evaluated based on a DNA standard curve. A part of the virus genome was amplified (primers in supplementary table S1) and purified by E.Z.N.A.® Cycle-Pure Kit (Omega, USA). The partial sequences of IAPV and SBPV by Sanger sequencing (LGC genomics, Germany) showed the highest identity to nt (nucleotide) reference EU436423.1 (IAPV) and GU938761.1 (SBPV, Harpenden strain), respectively. The concentration of purified templates was measured by Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, USA). The concentration was calculated to gcn per µl by the online tool (URL: http://cels.uri.edu/gsc/cndna.html; Accessed date: 20/June/2014). A serial 10 times dilution of the templates was made to obtain a standard curve for each virus by qPCR. The normalized gcn of each sample was represented by the ratio of the gcn calculated based on the DNA standard curve and the normalization factor from the internal reference gene peptidylprolyl isomerase A (*PPIA*) (Niu et al., 2014a) with the framework of qBase (Hellemans et al., 2007).

2.5. The expression of core genes of the siRNA pathway upon viral infections

The expression of key components, *Dicer-2* and *Ago-2*, of the siRNA pathway was measured on the above collected samples used for detecting virus gcn and PBS injected bees in all different time points served as controls. *PPIA* was used as internal control to normalize qPCR data as it was validated as the most stable reference gene cross different viral infection times and tissues (Niu et al., 2014a). Four to five biological replicates were included in each time point for virus

and PBS, respectively. The fold change of the mRNA level at each time point was given as the ratio of the relative gene expressions of the virus treated samples over the PBS controls collected at the same time point. All the experiments were performed on RNA extractions from the whole body. Extra experiments were performed on the RNA extracted from the abdomen, such as, abdomen was used in additional samples of SBPV and PBS injected bees at 3 dpi to measure *Dicer-2* expression. To investigate different viral inoculations in relation with *Dicer-2* expression, an amount of ~10^8 IAPV particles (mixed with sugar water instantly before feeding) was ingested per bee of two days old, and the feeding of PBS with the same condition served as a control. Subsequently, RNA samples were collected after 9 days for analyzing gene expression.

2.6. RNA isolation, cDNA synthesis, and qPCR

Extra RLT buffer (1.5~2 ml) was used to homogenize the lysed bumblebee tissues by mortar and the supernatant was centrifuged for three times to remove the deposit, then it was followed by the protocol of RNeasy mini kit (Qiagen, Germany). Afterwards, RNA was treated by TURBO DNA-free™ kit (Ambion, USA) to remove possible genomic DNA contamination. RNA quantity and quality were checked by Nanodrop and electrophoresis on 1.5% agarose gel. Two microgram of RNA was used to synthesize the cDNA by SuperScript® II Reverse Transcriptase (Invitrogen, USA) using oligo (dT) primers. To make sure that genomic DNA was removed we checked cDNA samples by PCR with exon spanning primers for RPL23 (supplementary data Table S1). The cDNA should produce an amplicon of 143 bp while the presence of genomic DNA will produce an extra amplicon of 452 bp. The qPCR was performed on a CFX96™ Real-Time PCR Detection using GoTaq® qPCR master (Promega, USA). Each reaction was performed in duplicate. The amplification specificities of primers were checked by both electrophoresis of the RT-PCR products and analysis of the dissociation curve. In addition, the

RT-PCR products were sequenced in order to confirm the amplification specificities. The qPCR data was normalized by suitable reference genes based on (amplification efficiency)$^{-\text{Delta Delta Cq}}$ in qBase^{PLUS}.

2.7. Small RNA libraries, sequencing and data analysis of virus-derived siRNA

The RNA samples were collected from whole body of 2 dpi IAPV (20 particles per bee) and SBPV (20,000 particles per bee) for small RNA sequencing. The PBS injected bees was also included as control. For each virus and control, four biological replicates were sequenced by the NXTGNT sequencing platform from the Ghent University. Concentration and quality of the total extracted RNA was checked by the Quant-iT^{TM} RiboGreen$^{®}$ RNA assay kit (Invitrogen, USA) and the RNA 6000 pico chip (Agilent Technologies, USA). Subsequently, one microgram of total RNA was used to start the library preparation by TailorMix miRNA Sample Preparation Kit v7 (SeqMatic, USA). Library preparation was carried out according to the manufacturer's instructions. The tRNA was added as carrier to minimize the loss of RNA via tube interaction. Libraries were quantified by qPCR, according to Illumina's protocol 'Sequencing Library qPCR Quantification protocol guide' (version February 2011). A high sensitivity DNA chip (Agilent Technologies, USA) was used to control the library's size distribution and quality. Single-end index 50 bp sequencing was performed on an Illumina MiSeq sequencer by loading 7 pM of each sample on the flowcell. A 10% PhiX spike-in was added as control.

Ambiguous and low quality bases and adaptor sequences were trimmed from the sequencing reads using CLC Genomics Workbench 7.0.2. No ambiguous bases were allowed and a quality setting of 0.05 was applied. Reads smaller than 15 bp after trimming or reads containing more than 10% of bases with Phred quality score lower than 20 were filtered with CLC Genomics

Workbench 7.0.2 and fastX-toolkit 0.0.13.1, respectively. The distribution of vsiRNAs was obtained by mapping reads to the IAPV (EU436423.1) and SBPV (GU938761.1) nt reference genomes using Bowtie 1.0.0 in 'best' mode with a seed length of 20. Maximum one mismatch was allowed in the seed, and two mismatches in the whole read sequence.

2.8. DsRNA synthesis and gene silencing optimization

A fragment of each target gene was amplified by PCR with target gene sequence specific primers plus T7 promoters (primers in supplementary table S1). These partial DNA templates of each gene were purified by E.Z.N.A.® Cycle-Pure Kit (Omega, USA), and were sequenced in order to confirm their specific amplifications. Then, one microgram of template was used to synthesize dsRNA according to the guideline of MEGAscript® RNAi Kit (Invitrogen, USA). The concentration and quality of each dsRNA were verified by Nanodrop and electrophoresis on 1.5% agarose gel. Based on experiments on successful silencing of six different genes, inlcuidng *Hop*, *PPIA*, *Dicer-1*, *Dicer-2*, *Sid*, and *Vago* (supplementary data Figure S1). We found out that 20 µg dsRNA in 20 µl /injection per bee/post 2 days injection can obtain effective gene silencing. The dsRNA was injected in the side of soft white-like cuticle between the 1st and 2nd abdominal segments into five to eight day old workers. The abdomen of each individual was collected for RNA isolation. The same procedure for dsGFP injection served as a negative control. The mock (no treatment) and elution buffer (provided by the dsRNA synthesis kit) were included as the control to check the possible effect of pure injection and dsGFP, respectively.

2.9. The effect of silencing *Dicer-2* on the RNAi efficiency and virus infection

We used “RNAi of RNAi” approach to investigate whether silencing of *Dicer-2* influenced the RNAi efficiency. First we silenced *Dicer-2* by injection of dsDicer2 (dsGFP serves as a control). After two days, the second injections were performed with dsPPIA (again with dsGFP as a

control) for silencing *PPIA*. Subsequently, the samples were collected for RNA extractions. The silencing efficiency of *PPIA* was used to evaluate if pre-silencing of *Dicer-2* was biologically significant to influence the later gene silencing efficiency. Two reference genes, RPL23 and UBI, were used to normalize the expression of *PPIA* as the most stable reference gene (Niu et al., 2014a) by qBase (Hellemans et al., 2007). A similar approach was applied to check the effect of pre-silencing of *Dicer-2* on the amount of viral gcn.

3. Results

3.1. High mortality after IAPV injection and low mortality after SBPV injection

IAPV caused extremely high mortality regardless of the amount of viral particles injected (Figure 1A), as there was no significant difference among 20, 200, and 2,000 particles injected (n=15~20 per treatment). All the IAPV injected bees died within 8 days, while PBS injection caused no mortality. In addition, feeding of high IAPV dose ($1x10^8$) also led to a ~80% mortality within two weeks (supplementary data Figure S2). In contrast, SBPV showed a marginal mortality of less than 30% after injection of 200,000 virus particles (Log Rank $p<0.046$, Breslow $p<0.045$). Injection of lower viral amounts (20,000 and 2,000 SBPV particles) resulted in few dead bees, which showed no significant difference in mortality with the PBS control (Fig 1B).

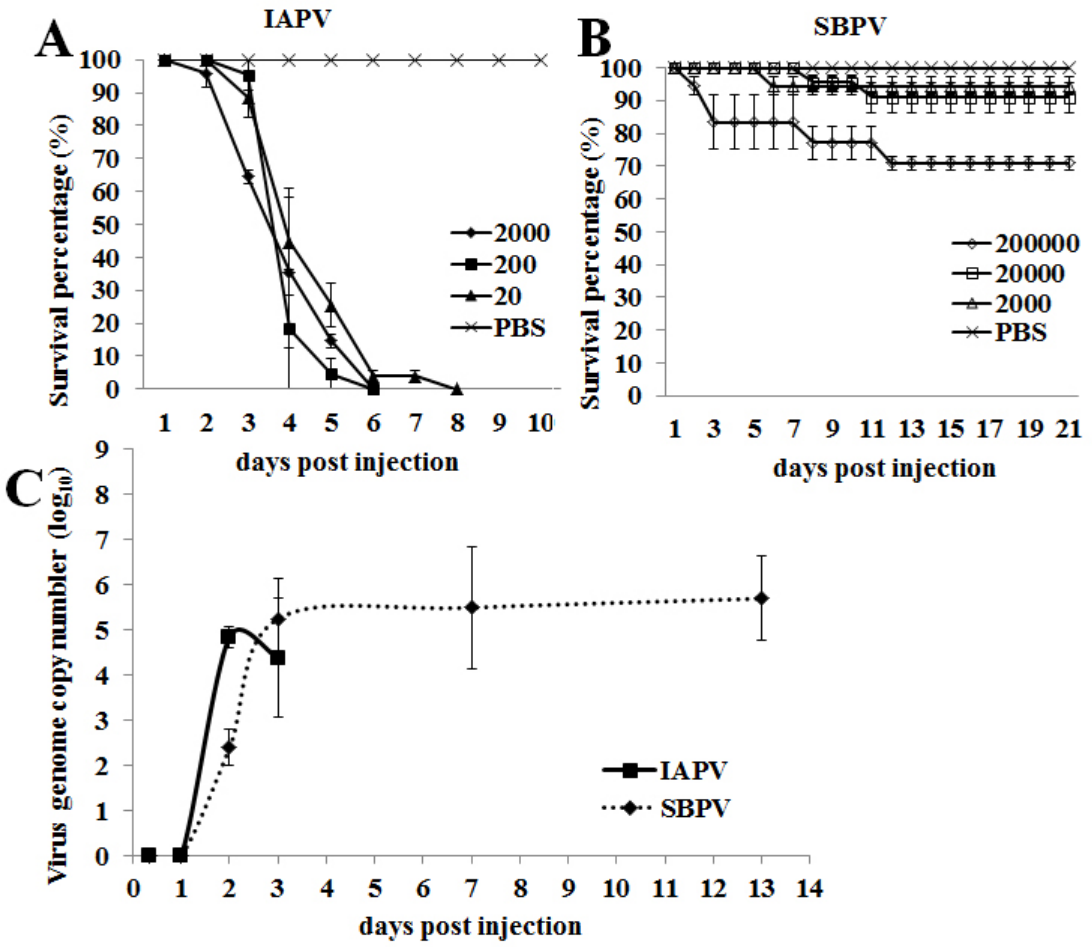


Figure 1. Survival percentage of bumblebees upon different viral injection doses and the genome copy number (gcn) during infection period.

(A): survival percentage of bumblebees upon IAPV injection (doses ranging from 20~2,000 particles). (B): survival percentage of bumblebees upon SBPV injection (doses ranging from 2,000 ~ 200,000 particles). Age fixed adult bees were used to perform injections and 15~20 biological replicates were included in each dose injection. PBS injected bees were used as control. The mean of survival percentage was based on three repeats and the error bar represented the standard error of mean. (C): quantification of IAPV and SBPV gcn during infection period. A number of 20 and 20,000 particles were injected per bee for IAPV and SBPV, respectively. The means of each gcn were represented based on Log_{10} transformation and the error bar represented the standard error of mean.

3.2. Analysis of reference gene stability in bumblebee

For the 1st series of experiments, the *M* values calculated by GeNorm^{PLUS} were less than 0.5 for all of the five candidate reference genes in whole bodies, guts and remnants, while in the heads the *M* values of three of the candidate genes (*RPL23*, *UBI* and *ELF1α*) were less than 0.5 (Table 1). When analyzing RT-PCR data from all the samples in 1st series together, all reference genes had an *M* value higher than 0.5 and ranked from the most stable (lowest *M* value) to the least stable (highest *M* value) as: *PPIA<TBP<RPL23<UBI<ELF1α* (Table 1). For the 2nd series of experiments, concerning the virus-infection time course, we analyzed the stability of the reference genes at 8, 24, 48, 72 h after injecting the bumblebees with IAPV. The IAPV transcript levels in the 8 h and 24 h post-inoculation samples were below the detection limit of RT-PCR and comprised the 'low IAPV' samples, while the 48 h and 72 h post-inoculation samples comprised the 'high IAPV' samples (Figure 2A – IAPV inoculation). The IAPV detection limit ($1x10^5$ particles/bee) was determined by spiking a virus-free bumblebee extract with 1×10^7 IAPV particles, purifying the RNA and preparing a ten-fold serial dilution of the RNA prior to RT-PCR (Figure 2A – IAPV particles). This detection limit corresponds to ~2 IAPV cDNA molecules per PCR reaction (*i.e.* close to the theoretical detection limit of PCR), after accounting for the different dilution factors associated with RNA extraction, DNAse treatment, cDNA synthesis and PCR. As shown in Table 1, at 8, 48 and 72 h post-inoculation, most of the candidate reference genes were relatively stable, with *M* values less than 0.5. Although at 24 h post-inoculation the *M* values of all candidate reference genes were elevated relative to the other time-points, while that of *PPIA* stayed below 0.5. When combining all IAPV- and control-injected samples into a single analysis, *PPIA*, *RPL23* and *UBI* were the most stable reference genes, all with *M* values below 0.5 (Table 1). To assess the effect of virus infection on reference gene

stability, we combined only the virus infected samples from all time points into a single analysis. These results indicated that *RPL23* and *PPIA* were the two most stable internal reference genes.

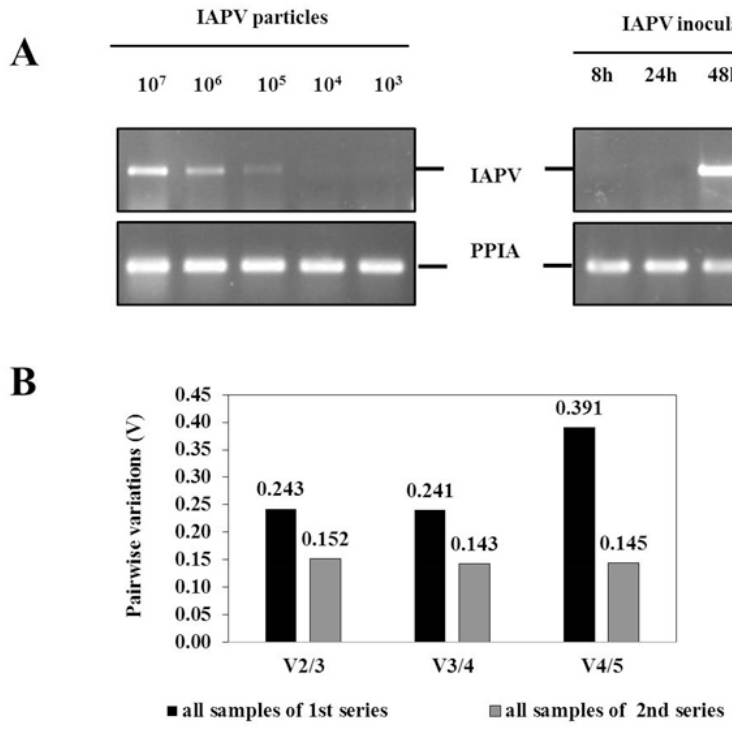


Figure 2. IAPV detection and determination of optimal number of reference genes.

(A) left: IAPV detection threshold determination using dilution series of IAPV particles; right: IAPV detection at different post-injection time points. (B) Determination of optimal number of reference genes for all samples of each series.

The results from the data analysis using GeNorm^{PLUS} were compared with those obtained using NormFinder, an alternative program for analyzing the stability of potential internal reference genes. The rankings of the five candidate internal reference genes for individual body parts (1st series) and individual post-inoculation time points (2nd series) slightly differ between the two analyses except the whole body group (Table 1). This in itself is not unexpected, since the two analyses use different algorithms. However, both algorithms identify *PPIA* and *RPL23* as the

best two internal reference genes for IAPV infection studies in bumblebees and these are also among the top three genes identified by both algorithms for body parts-specific studies. One remarkable observation for the body parts-specific studies (1st series) was the stability values were (with a few exceptions) well below 0.5 for individual tissues or whole body extracts, but above 0.5 when the data was pooled into a single analysis indicated by GeNorm^{PLUS}. We therefore evaluated, using GeNorm^{PLUS}, whether the use of multiple reference genes could improve the normalization in such cases. This was done by calculating the pairwise variation $V_n/_{n+1}$, which measures the effect of adding extra reference gene on the normalization factor (the geometric mean of the expression values of selected reference genes). So the value of $V_n/_{n+1}$ was equal to the ratio of two sequential normalization factors through stepwise inclusion of more reference genes. None of the $V_n/_{n+1}$ values dropped below the recommended upper threshold of 0.15 (Vandesompele et al., 2002), suggesting that extra candidate reference genes are needed for stabilizing the normalization of RT-qPCR in gene expression analysis when grouping the data from various body parts and whole body extracts together. We also calculated the $V_n/_{n+1}$ for all samples of the 2nd series (Figure 2B), where bumblebees were analyzed at different time intervals after IAPV infection. The combined data of all IAPV-infected and control samples suggested that just three internal reference genes (*PPIA*, *RPL23* and *UBI*) were sufficient to normalize the data for all samples and that including a fourth reference gene would not improve normalization. To sum up, *PPIA* was the single most optimal internal reference gene for IAPV infection studies in bumblebees, due to its good stability at individual time intervals after virus infection, as well as for all time intervals combined, while the *PPIA*-*RPL23*-*UBI* combination was optimal and fully sufficient for normalization of IAPV infection experiments when using multiple reference genes.

Table 1. Ranking of the stability for the five candidate internal reference genes investigated in two different series of samples.

Method	First series: Samples of virus-free whole body and body parts of *Bombus terrestris*					
	Ranking	Whole Body	Head	Gut	Remnants	Whole Body/Head/Gut/Remnants
GeNorm PLUS	1	PPIA (0.194)	RPL23 (0.300)	RPL23 (0.145)	PPIA (0.195)	PPIA (0.652)
	2	TBP (0.196)	UBI (0.346)	PPIA (0.151)	TBP (0.213)	TBP (0.655)
	3	ELF1α (0.220)	ELF1α (0.400)	TBP (0.164)	RPL23 (0.244)	RPL23 (0.696)
	4	RPL23 (0.282)	PPIA (0.542)	UBI (0.196)	UBI (0.389)	UBI (0.857)
	5	UBI (0.401)	TBP (0.704)	ELF1α (0.347)	ELF1α (0.493)	ELF1α (1.326)
Normfinder	1	TBP (0.115)	RPL23 (0.005)	RPL23 (0.057)	PPIA (0.027)	TBP (0.173)
	2	UBI(0.144)	TBP (0.018)	ELF1α (0.061)	RPL23 (0.027)	RPL23(0.241)
	3	RPL23 (0.207)	PPIA (0.036)	PPIA (0.071)	TBP (0.155)	PPIA (0.339)
	4	ELF1α (0.235)	UBI(0.159)	TBP (0.081)	UBI(0.398)	UBI(0.357)
	5	PPIA (0.260)	ELF1α (0.706)	UBI(0.123)	ELF1α (0.400)	ELF1α (0.669)
	Second series: Samples of whole bodies of *Bombus terrestris* at different time intervals after IAPV infection					
	Ranking	**8 h**	**24 h**	**48 h**	**72 h**	**8/24/48/72 h**
GeNorm PLUS	1	UBI (0.278)	PPIA (0.426)	PPIA (0.400)	UBI (0.316)	PPIA (0.483)
	2	ELF1α (0.301)	TBP (0.511)	RPL23 (0.400)	RPL23 (0.319)	RPL23 (0.491)
	3	RPL23 (0.309)	RPL23 (0.565)	ELF1α (0.402)	PPIA (0.359)	UBI (0.498)
	4	PPIA (0.388)	UBI (0.632)	UBI (0.473)	TBP (0.468)	ELF1α (0.566)
	5	TBP (0.516)	ELF1α (0.722)	TBP (0.520)	ELF1α (0.622)	TBP (0.651)
Normfinder	1	RPL23 (0.075)	PPIA(0.016)	RPL23 (0.059)	PPIA (0.129)	PPIA (0.138)
	2	PPIA (0.079)	RPL23(0.089)	PPIA (0.061)	RPL23 (0.192)	RPL23 (0.262)
	3	UBI(0.150)	UBI(0.106)	UBI(0.180)	ELF1α (0.210)	UBI(0.289)
	4	ELF1α (0.164)	ELF1α (0.331)	ELF1α (0.209)	UBI(0.213)	ELF1α (0.385)
	5	TBP (0.274)	TBP (0.344)	TBP (0.345)	TBP(0.575)	TBP (0.496)

The data were a nalyzed by both GeNorm^{PLUS} and Normfinder, The stability values are given between parentheses.

3.3. Fast replication of IAPV and SBPV after injection

To know the replication dynamics of viruses after injection into bees, we checked viral gcn at various time points. Based on the standard curves of the two viruses (supplementary data Figure S3), we calculated viral gcn at different time points, i.e. 8 hours, 1, 2, and 3 days for IAPV, and 8 hours, 1, 2, 3, 7, and 13 days for SBPV (Figure 1C). Within one dpi we could not yet detect IAPV (n=8) and SBPV (n=9). Our theoretical detection limit was around 10^4 viral particles since the sample collection through the processes of RNA extraction, DNase treatment, cDNA prep and qPCR (Niu et al., 2014a). After one dpi, the gcn for both viruses rapidly increased. IAPV reached the highest gcn within two days, while it took longer for SBPV to reach the highest plateau. At two dpi, the viral gcn of IAPV was already ~284 times higher than that in SBPV at two dpi (t-test: t=-5.521, df=6, *p=0.001*).

Bees injected with 20,000 particles of SBPV showed no mortality (Figure 1B) but the relative viral load in these infected bees reached a plateau around three dpi (Figure 1C). Based on viral detection by RT-PCR, lower dosage of SBPV (less than 2,000 particles) was not able to successfully infect the host in our study (data not shown). In contrast, IAPV acts as a very virulent virus to bumblebees after injection, which showed a noticeable mortality already after two dpi. It should be pointed out that the mortality between 2 and 3 dpi could somehow influence the detection of IAPV gcn at samples collected in 3 dpi. We could only use bees which were still alive at 3 dpi to detect viral gcn because we could not perform normalization with stable internal reference gene in dead bees. Therefore, we speculated that IAPV could still reach higher titers in bumblebees. IAPV gcn was sharply increased between 1 and 2 dpi, extra time points within this period could improve more detailed characterizations of IAPV replication dynamics.

3.4. Induction of *Dicer-2* expression after infection with virulent IAPV and avirulent SBPV

Next, we followed the gene expression of two key components (*Dicer-2* and *Ago-2*) of the siRNA pathway. The fold change of each gene expression at each dpi (Figure 3) was represented by the ratio of the normalized expression of the gene in the virus injected bees over the PBS injected bees. For each dpi we had virus injected bees (n =4~5) and PBS injected bees (n=4~5). There was a significant induction (t-test: t=9.127, df=6, $p<0.001$) of *Dicer-2* expression (~3.3 fold) at 2 dpi of IAPV on the level of whole body (Figure 3A). We did not detect a significant induction of *Dicer-2* expression after SBPV infection at level of whole body, but a significant induction (t-test: t=-5.060, df=14, $p<0.001$) of *Dicer-2* was detected in the abdomen 3 dpi of SBPV (n=8) (Figure 4A). Upon IAPV feeding (n=8) we also detected a significant upregulation (t-test: t=3.853, df=9.5, $p=0.004$) of *Dicer-2* (Figure 4B). However, the expression of *Ago-2* upon the infection of two viruses was stable in all detected time points (Figure 3B).

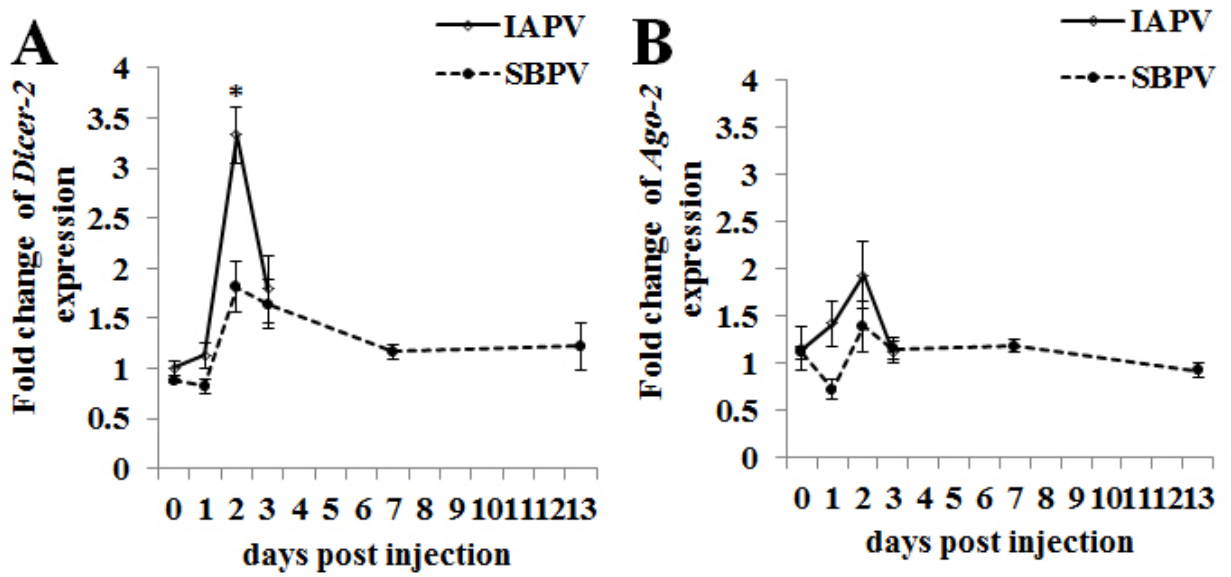


Figure 3. Fold changes of two core genes in the siRNA pathway upon IAPV and SBPV infection.

(A): fold change of *Dicer-2* expression upon viral infections; (B): fold change of *Ago-2* expression upon viral infections. A number of 20 and 20,000 particles were injected per bee for IAPV and SBPV, respectively. The fold changes of gene expression were equal to the ratio of the relative expression of each gene in virus infected samples over the relative expression of this gene in control samples (PBS injected bees). The relative expression of each gene was calculated based on internal reference gene *PPIA*. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p< 0.05$ was labelled with asterisks (*).

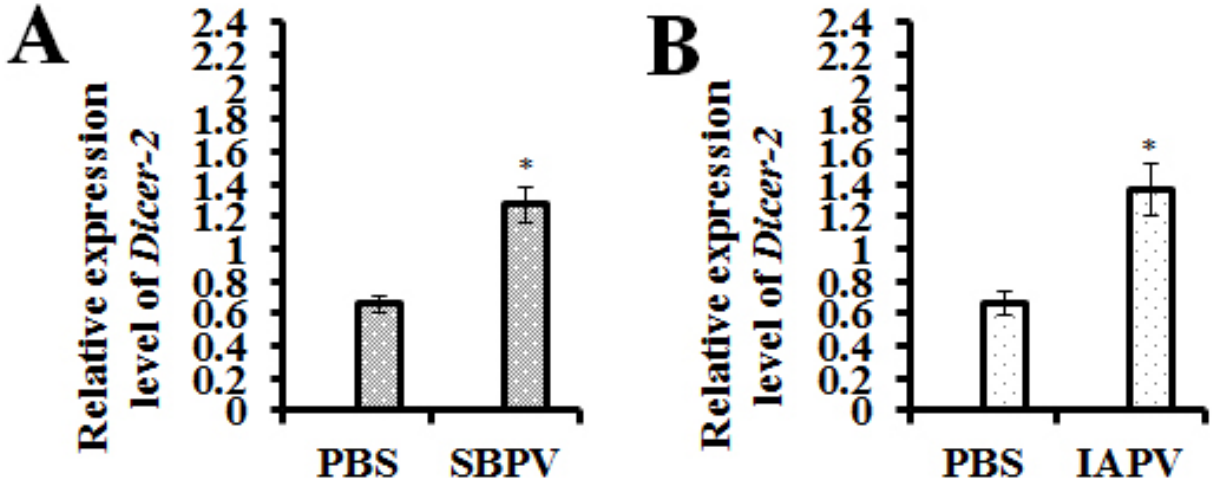


Figure 4. The relative expression of *Dicer-2*.

(A): *Dicer-2* expression in the abdomen of SBPV injected bees. An amount of 20,000 particles SBPV were injected per bee and the abdomen of each individual was collected at 3 dpi for RNA extraction. The same procedure for PBS injected bees was used as a control. (B): *Dicer-2* expression in IAPV infected bees through feeding. An amount of ~10^8 particles IAPV (mixed with sugar water instantly before feeding) was ingested per bee. Post 9 days of IAPV feeding, RNA was isolated from the abdomens of individual bees, and those fed with PBS served as the control.). The relative expression of each gene was calculated based on internal reference gene *PPIA*. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p< 0.05$ was labelled with asterisks (*).

3.5. Production of vsiRNAs during virus infection

In order to identify further evidences of the siRNA pathway activity, we sequenced the small RNAs of viral infected samples. Our results showed that in IAPV infected samples the range of the proportion of vsiRNA among the total small RNAs was 1.5~21.6%, which was 0.1~1.3% in SBPV infected samples (Table 1). Looking at the length distribution of vsiRNAs, it exhibited a high peak at 22 nt-long vsiRNAs in all IAPV infected samples, which may represent as Dicer-2 products (Figure 4A). Intriguingly, the increase of 22 nt-long vsiRNAs derived from IAPV antigenome but not genome showed the correlation with virus titers in four biological controls of IAPV infected samples. For SBPV, the peak at 22 nt-long vsiRNAs was not observed and only few antigenome derived vsiRNA were detected (Figure 4B), and the distribution of 18~27 nt-long vsiRANs of SBPV mainly derived from genome (supplementary data Figure S3). The results also showed that IAPV vsiRNAs (Figure 5 and supplementary data Figure S4) covered most regions of the viral genome and antigenome. We also observed some vsiRNAs with high counts. These observed high counts were not artifacts of small fragments of host RNA with high similarity to the IAPV genome because the small RNAs of SBPV and PBS samples did not show similar peaks when mapping to IAPV genomes.

Table 2: The number of total sequenced small RNAs (sRNAs) and virus-derived sRNAs (vsRNAs).

Sample	Number of sRNA (*)	Number of vsRNAs (IAPV or SBPV)	vsRNA/ sRNA (%)
IAPV1	775,032	167,230	21.6
IAPV2	788,819	11,451	1.5
IAPV3	764,809	16,998	2.2
IAPV4	807,746	65,502	8.1
SBPV1	633,488	606	0.1
SBPV2	577,622	5,873	1.0
SBPV3	911,559	1,072	0.1
SBPV4	791,227	10,178	1.3

(*) total siRNAs after clean-up, i.e. after adapter and quality trimming and rRNA/tRNA filteri

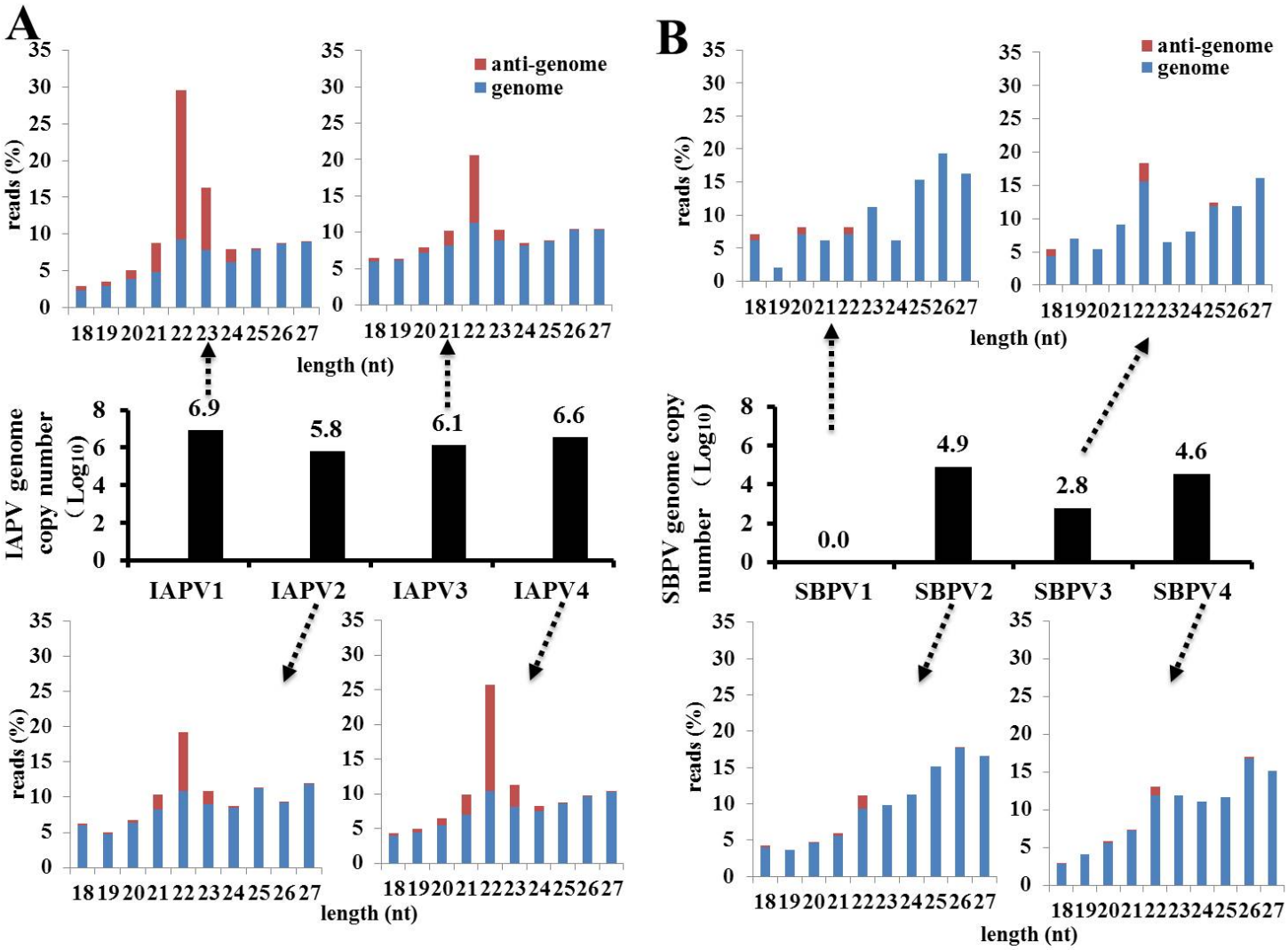


Figure 5. The distribution (in length: percentage of total number of virus-derived small RNAs) of virus-derived small RNAs.

(A): length distribution of IAPV-derived small RNAs and viral genome copy number in each biological replicate; (B): length distribution of SBPV-derived small RNAs and viral genome copy number in each biological replicates. The sequenced samples were collected from whole body at 2 dpi injection of IAPV (20 particles per bee) and SBPV (20,000 particles per bee). The gcn in each sample was represented by Log_{10} transformation. The sample SBPV1 had the least gcn (the absolute value was very close to 0 based on standard curves), but the status of infection by SBPV could be detected by RT-PCR in this sample.

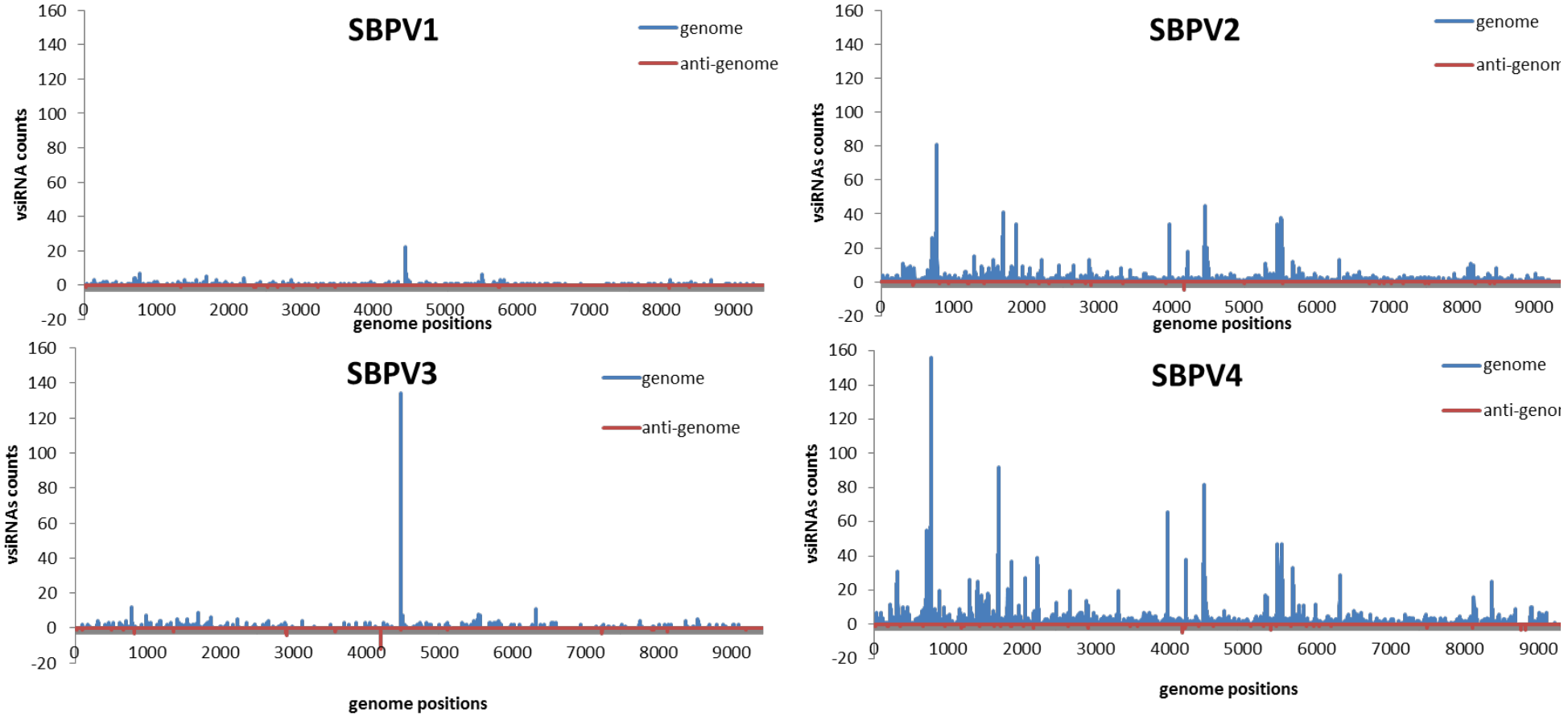


Figure 6: distribution of 18~27nt vsiRNA on SBPV genome and antigenome.

The sequenced samples were collected from whole body at 2 dpi injection of SBPV (20,000 particles per bee). The counts of vsiRNAs derived from SBPV genome represented by positive number and in blue bars, and for those derived from SBPV antigenome represented by negative number and in red bars.

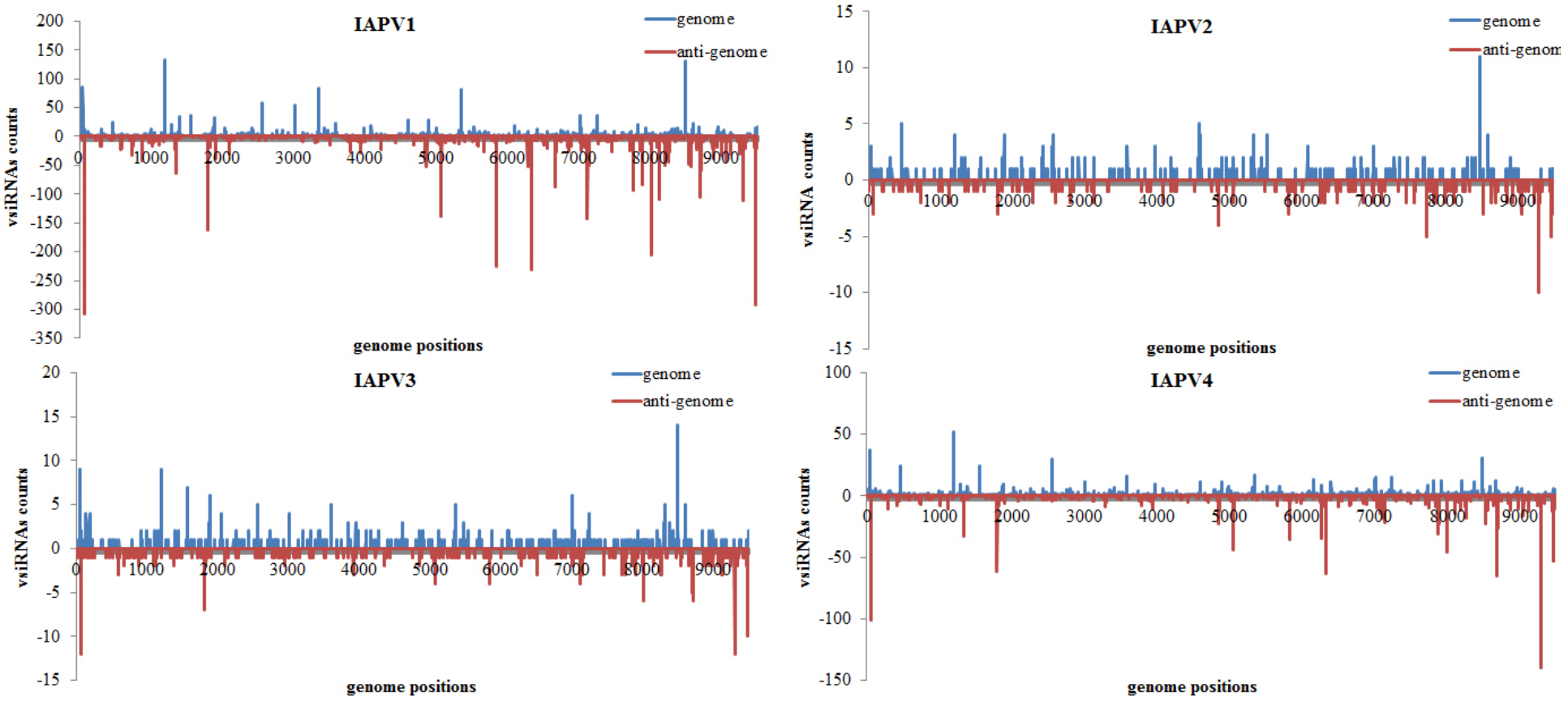


Figure 7. The distribution of ~22 nt-long small RNAs (vsiRNA) on regions of IAPV genome and antigenome.

The sequenced samples were collected from whole body at 2 dpi injection of IAPV (20 particles per bee). The counts of vsiRNAs derived from IAPV genome represented by positive number and in blue bars, and for those derived from IAPV antigenome represented by negative number and in red bars.

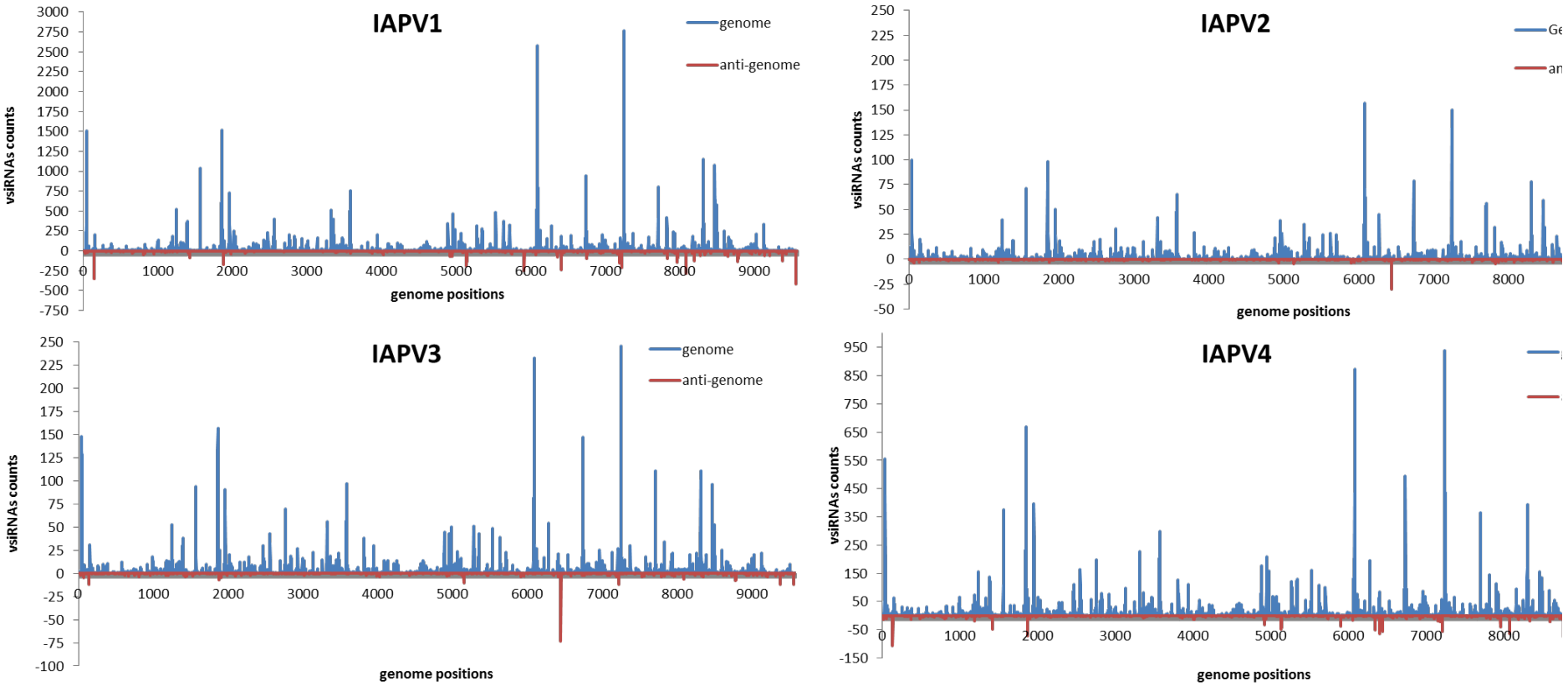


Figure 8: distribution of 18~27nt vsiRNA on IAPV genome and antigenome.

The sequenced samples were collected from whole body at 2 dpi injection of IAPV (20 particles per bee). The counts of vsiRNAs derived from IAPV genome represented by positive number and in blue bars, and for those derived from IAPV antigenome represented by negative number and in red bars.

3.6. *Dicer-2* silencing

The amount of dsRNA required to sufficiently silence genes in bumblebees was optimized in pre-experiments. It turned out that a high dose of dsRNA was needed (~20 μg). The high dose of dsRNA used to silence genes was also reported in some experiments of bumblebees and honeybees (see table 1 in review (Niu et al., 2014b)). Compared with dsGFP treatment, we could get ~60% down-regulation of *Dicer-2* (Figure 6A: silencing effect). However, this result was complex: injection of dsGFP can actually induce the expression of *Dicer-2* up to 43 or 50% compared with the injection of elution buffer or mock (Figure 6A: induction effect), respectively. A same level of induction to *Dicer-2* was also observed by injection of dsSBPV (SBPV sequence-specific dsRNA) or by injection of dsPPIA which can silence host gene *PPIA* (Figure 6A: induction effect). Therefore, in general, dsRNA (such as random control gene: dsGFP, virus: dsSBPV, or host: dsPPIA) induced the transcriptional level of *Dicer-2*. However, in the case of injection of dsDicer2, the expression of *Dicer-2* was significantly lower compared with injection of dsGFP, dsSBPV or dsPPIA, but not significantly different with the mock or elution buffer injected groups (ANOVA: $F=15.191$, $p<0.001$, multiple comparisons- Tukey HSD). It has to be admitted that, silencing of *Dicer-2* can reach ~60% depletion of its expression in our setup, but biologically, it is as the same level as normal conditions (mock or elution buffer). Therefore, our "silencing of *Dicer-2*" should only be regarded in the context of the appropriate control-dsGFP.

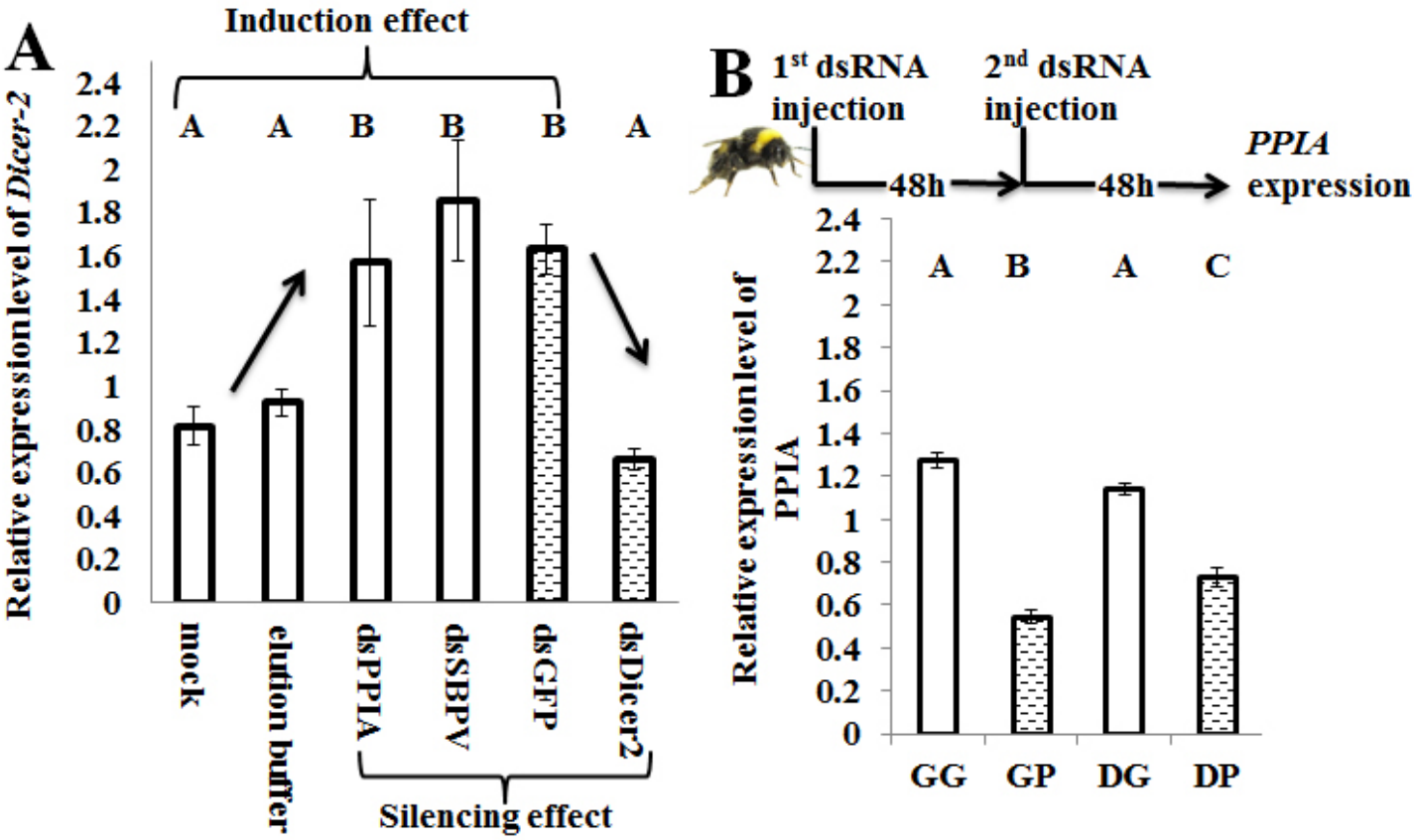


Figure 9. Silencing of *Dicer-2* and its effect on RNAi efficiency.

(A): expression of *Dicer-2* after injection of different dsRNAs. A total of 20 µg (in 20 µl) of dsRNA was injected per bee, and a same dose of dsGFP served as control. The abdomen of each individual was collected for RNA isolation. The mock (no treatment) and elution buffer (provided by the dsRNA synthesis kit) were included as the control for effect of pure injection and dsRNA injection, respectively. DsDicer-2 (*Dicer-2* sequence-specific dsRNA) was the silencing treatment, and dsGFP, dsSBPV (SBPV sequence-specific dsRNA) and dsPPIA (*PPIA* sequence-specific dsRNA) were used as controls. The expression of *Dicer-2* was normalized by reference genes RPL23 and UBI. The significant difference of *Dicer-2* expression ($p<0.05$) among different treatments were represented by different capital letters (ANOVA: F=15.191, $p<0.000$, multiple comparisons-Tukey HSD). Compared with mock and elution buffer, the injections of dsPPIA, dsSBPV, and dsGFP could induce the expression of *Dicer-2*, referred as "induction effect". In comparison with other dsRNA injections, the injection of dsDicer-2 could reduce the expression of *Dicer-2*, referred as "silencing effect". (B): silencing efficiency of *PPIA* through pre-silencing of *Dicer-2* (1st injeciton-2nd injection, GG: dsGFP-dsGFP; GP: dsGFP-dsPPIA; DG: dsDicer2-dsGFP; DP: dsDicer2-dsPPIA (n=5). The significant difference ($p<0.05$) among different treatments were represented by different capital letters (ANOVA: F=84.062, $p<0.001$, multiple comparisons-Tukey HSD): $p = 0.015$ (GP vs. DP), $p = 0.08$ (GG vs. DG), $p < 0.001$ for other comparisons).

3.7. Influence to gene silencing efficiency and virus infection through pre-silencing of *Dicer-2*

This experiment was conducted to test whether the silencing of *Dicer-2* could inhibit the RNAi efficiency. Two injections were included: the *Dicer-2* silencing was achieved through the 1st injection and the later *PPIA* silencing (PPIA as the indicator) was performed by the 2nd injection. This design created four treatments (1st injection-2nd injection): dsGFP-dsGFP, dsGFP-dsPPIA, dsDicer2-dsGFP, and dsDicer2-dsPPIA (n=5), in which we measured *PPIA* expression at two days after the 2nd injection (Figure 6B) (ANOVA: F=84.062, $p<0.001$, p value for each comparison calculated by Tukey HSD). First, we noticed a significant silencing of *PPIA* in *Dicer-2* pre-silenced samples (dsDicer2-dsGFP vs. dsDicer2-dsPPIA: $p<0.001$) and non-pre-silenced samples (dsGFP-dsGFP vs. dsGFP-dsPPIA: $p<0.001$). This could be regarded as a successful *PPIA* silencing in bumblebees. A second control was the stable expression of *PPIA* when comparing dsGFP-dsGFP and dsDicer2-dsGFP, meaning that the injection of dsDicer2 had no effect on the expression of *PPIA*. Therefore, the significant lower expression of ~21% of *PPIA* in dsGFP-dsPPIA compared with dsDicer2-dsPPIA ($p=0.015$) was a consequence of the pre-silencing of *Dicer-2* (Figure 6B). This result indicated the pre-silencing of *Dicer-2* could influence RNAi efficiency. Under this basis, we tested whether *Dicer-2* silencing could influence the amount of viral gcn. By performing the same "RNAi of RNAi" strategy, the gcn level was 5.04 and 5.11 on dsGFP and dsDicer2 injected samples (n=17-18) for IAPV (Figure 7A), respectively. It was 1.51 and 1.34 for the SBPV (figure 7B) gcn on dsGFP and dsDicer2 injected samples (n=11~12). For both viruses, compared with the control groups of dsGFP, pre-silencing of *Dicer-2* did not significantly influence the amount of viral gcn (Figure 7).

Although pre-silencing of *Dicer-2* (~60%) was achieved before the viral inoculations, virus

infection could also influence the expression of *Dicer-2*. Indeed, the expression of *Dicer-2* was induced by IAPV and SBPV infections. Our results showed the remaining *Dicer-2* silencing proficiency was 23.5% with significant difference in dsGFP compared with dsDicer2 (T-test: t=3.043, df=30, *p=0.005*) in IAPV infected samples, and the remaining *Dicer-2* silencing proficiency was 49.5% with significant difference in dsGFP compared with dsDicer2 (T-test: t=4.419, df=14.354, p=*0.001*) in SBPV infected samples (supplementary data Table S2). This reduced *Dicer-2* silencing proficiency compared with ~60% pre-silencing may be partially influenced by reduced silencing effects as longer time of post injection of dsRNA.

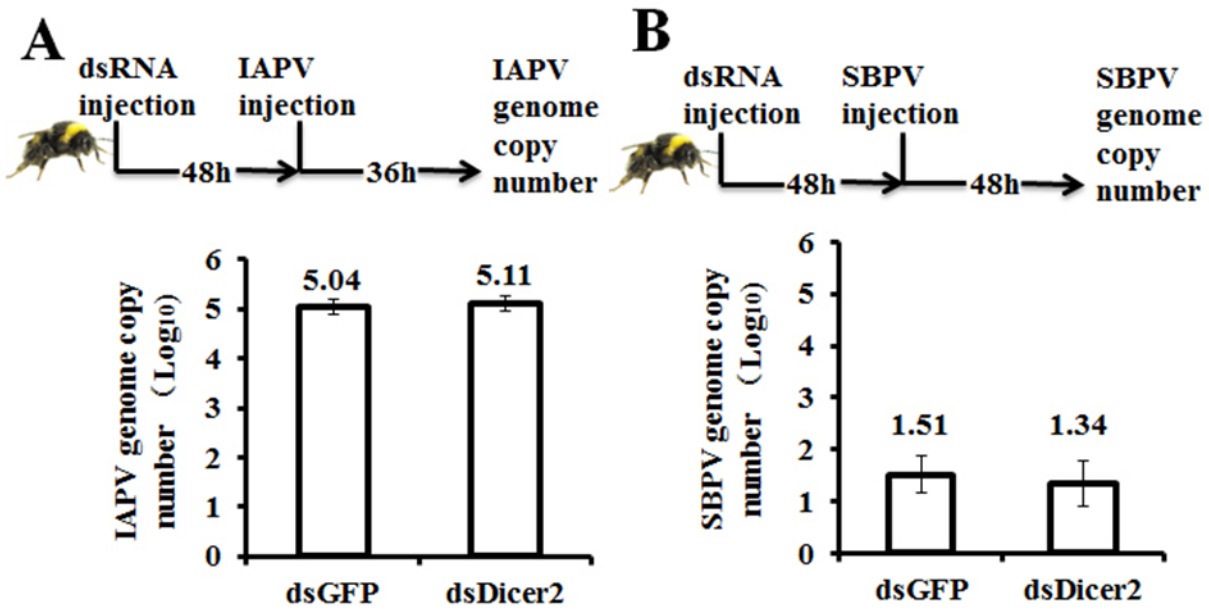


Figure 10. Genome copy numbers (gcn) of viruses after silencing of *Dicer-2*.

(A): IAPV gcn after silencing of *Dicer-2*. Firstly, dsDicer2 was injected to silence *Dicer-2*, post 48h injection, then IAPV (20 particles per bee) was injected to inoculate bees. Subsequently, RNA was collected post 36h injection of IAPV for measuring viral gcn. DsGFP injection was included as control; (B): SBPV gcn upon pre-silencing of *Dicer*-2. Firstly dsDicer2 was injected to silence *Dicer-2*, post 48h injection, then SBPV was injected to inoculate bees. Subsequently, RNA was collected post 48h injection of SBPV for measuring viral gcn. DsGFP injection was included as control. The means of each gcn were represented based on Log_{10} transformation and the error bar represented the standard error of mean. The level of significance was tested by T-test.

Table 3: *Dicer-2* expressions in the samples collected to detect the effect of pre-silencing of *Dicer-2* on viral genome copy numbers (samples associated with Figure 7)

Experiment	Treatment	Relative expression *Dicer-2* (mean ± sem)	Test	Remaining *Dicer-2* silencing proficiency
Pre-silencing *Dicer-2* to test effect on IAPV	dsGFP-IAPV	1.13 ± 0.08	(T-test: t=3.043, df=30, p=0.005)	23.52%
	dsDicer2-IAPV	0.87 ± 0.04		
Pre-silencing *Dicer-2* to test effect on SBPV	dsGFP-SBPV	1.23 ± 0.12	(T-test: t=4.419, df=14.354, p=0.001)	49.44%
	dsDicer2-SBPV	0.62 ± 0.06		

4. Discussion

The immune response in insect is a cascade of protection levels within different organ systems and tissue layers (Buchon et al., 2014). Tissues like the gut in the digestive system, the Malpighian tubules in the excretory system, and trachea in the respiratory system, are the first barriers for the virus to break. Each organ system has its own local immunity trying to prevent the initial infection. Once the pathogen enters the circulatory system a systemic immune response will be activated. Bee viruses often infect multiple tissues without inducing obvious symptoms (covert infection pattern) in honeybees (Aubert et al., 2008). When the local immunity is passed, the systemic immunity will play an important role in the attenuation of viral replication to prevent the overflow from a covert infection toward a diseased bee with overt symptoms. In our current experimental setup, IAPV acts as a highly lethal infection in bumblebees, it replicates fast and kills the bee within a few days. In contrast, as a non-lethal infection, SBPV, replicates fast but slower compared to IAPV. Screenings of wild bumblebees have also retrieved these two viruses, but symptoms in wild bumblebees have not yet been reported (Fürst et al., 2014; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010). Here we investigated the involvement of the siRNA pathway for these two viruses. We found that both virus infections induced the transcriptional level of *Dicer-2* in the bumblebee. Intriguingly, we also detected the induction of *Dicer-2* expression by injection of different dsRNAs. In bumblebee and honeybee, it is suggested that non-specific dsRNA can mediate certain antiviral responses, but the mechanism is still unclear (Flenniken and Andino, 2013; Piot et al., 2015). DsRNA, a pathogen-associated molecular pattern (PAMP) recognized by the host immunity system, can influence the expressions of genes in bees (Flenniken and Andino, 2013; Nunes et al., 2013). The up-

regulation of *Dicer-2* upon different dsRNAs (dsGFP/dsPPIA/dsSBPV) in the current study may suggest that the non-specific dsRNA mediated antiviral responses could be partially associated with the siRNA pathway. However, it remains to be investigated whether the expression of *Dicer-2* is induced because of a PAMP function of dsRNAs, or because of a positive feedback mechanism sensing the Dicer-2 processing in the host, or other unknown off-target effects of dsRNAs.

For RNA viruses, the major source cleaved by *Dicer-2* is viral-derived dsRNAs (Marques et al., 2013; Sabin et al., 2013; Siu et al., 2011). In honeybees, samples from the field and artificial infections were found to contain vsiRNAs with matches to IAPV, KBV, DWV and/or Varroa destructor virus-1 (Chejanovsky et al., 2014; Ryabov et al., 2014; Wang et al., 2013). However, only limited samples from wild bumblebees have been investigated, which showed no detectable vsiRNAs (Wang et al., 2013). In our artificially infected bumblebees we detected vsiRNAs from both IAPV and SBPV. Nevertheless, only samples infected with IAPV showed the typical predominant peak of 22 nt-long vsiRNAs which may suggest the Dicer-2 activity. We noticed that 22 nt-long of vsiRNAs showed a high proportion of the antigenome. In the four biological replicates, the increase of 22 nt-long vsiRNAs derived from IAPV antigenome but not genome showed the correlation with virus titers. In other word, the higher IAPV gcn in sequenced sample, the higher proportion of 22 nt-long vsiRNAs derived from antigenome but not genome. This suggests the increased virus titer in infected bumblebees tends to generate more 22 nt-long vsiRNAs derived from antigenome than that from genome. Strikingly, in IAPV infected honeybees the 22 nt-long vsiRNA was also showed a high proportion from the antigenome (Chejanovsky et al., 2014). However, in DWV infected honeybees, it was shown that the number of 21~22 nt-long vsiRNAs originating from the genome were 3 to 4 times highly compared to

antigenome (Ryabov et al., 2014). The dominance of vsiRNAs with rather a genome orientation over an antigenome is widely reported in virus infected insects (Marques et al., 2013; Sabin et al., 2013; Schirtzinger et al., 2015). In some plant RNA viruses, it has already been observed that different viruses induced different vsiRNA polarity, which speculate that the mechanism of strand polarity would be virus specific rather than host dependent (Pantaleo et al., 2010). It is well-known that some dicistroviruses could inhibit the antiviral activity of the siRNA pathway (Nayak et al., 2010; van Rij et al., 2006). Interestingly, it has been suggested that IAPV encoded a putative viral suppressors of RNAi (VSR) to impair the honey bee antiviral response of RNAi (Chen et al., 2014). The existence of this VSR is based on the presence of a conserved protein cleavage site, encompassing the consensus octamer DvExNPGP in IAPV (Chen et al., 2014). Accordingly, the upstream RNA genome of this consensus octamer DvExNPGP encodes a protein, namely 1A, which, in two closely related viruses of IAPV, i.e. Drosophila C virus (DCV) and Cricket paralysis virus (CrPV), acts as viral VSRs (Nayak et al., 2010; van Rij et al., 2006). Specifically, DCV-1A inhibits the activity of Dicer-2 to generate vsiRNA from virus related dsRNAs (van Rij et al., 2006). Whereas, CrPV-1A acts through inhibition of the slicer activity of mature Ago-2 associated RISC (Nayak et al., 2010). However, it needs to be pointed out that the presence of VSR in IAPV is still speculative and is only based on *in silico* similarity because the biological evidence of VSR in IAPV (Chen et al., 2014) could also alternatively be explained by different target accessibility of the siRNAs used in their study to target different parts of viral genome (Kieft, 2008; Ren et al., 2012; Shao et al., 2007) or by effects on the initiation of whole mRNA degradation of the viral genome leading to no or less translation of viral polyproteins (Houseley and Tollervey, 2009; Niu et al., 2014b).

As an avirulent virus in the current study, SBPV infected samples showed the induction of

Dicer-2 expression, while we did not detect possible Dicer-2 processing products- 22 nt-long vsiRNAs. The detection of possible Dicer-2 processed vsiRNA could be influenced by the total amount of virus. For example, the amount of gcn in SBPV was much lower than that in sequenced samples of IAPV. Indeed, the percentage of vsiRNAs within the total sequenced siRNA was much lower than that from IAPV samples. The samples of SBPV in 3 dpi showed relatively equivalent viral gcn compared with samples of IAPV infection in 2 dpi, therefore, small RNA sequencing on these samples may be useful to see whether SBPV infection can lead to the production of ~22 nt-long vsiRNAs peak. However, with up to 10,178 of siRNAs in one of the SBPV sequenced samples which is relatively in the same level as one of the IAPV samples with 11,451 vsiRNAs (Table 1 and supplementary data Figure S3), it seems we have enough sequencing reads to see the results of Dicer-2 processing in SBPV infection if it would be present. The low number of vsiRNAs derived from SBPV may suggest us to explore possible SBPV encoded VSR in future studies.

The "RNAi of RNAi" approach resulted in a different *Dicer-2* expression of ~60% between the dsGFP and dsDicer2 treatments. This could significantly influence the silencing efficiency of the housekeeping gene *PPIA,* but we did not detect a significant influence to the amount of IAPV and SBPV gcn. Taken all evidences together for both viruses, there are several proposed explanations for not detecting an effect on viral gcn after pre-silencing of *Dicer-2*, 1) virus replication after injection in both dsGFP and dsDicer2 were so fast that the attenuation performed by ~60% differential expression of *Dicer-2* was almost negligible. We knew that viral infections could induce the expression of *Dicer-2* which might hamper some extent of the effect provided by pre-silencing of *Dicer-2*; 2) the undetermined activity downstream of the siRNA pathway, especially Ago-2 associated RISC could be more crucial in the case of IAPV infection;

Ago-2 another core component of the siRNA pathway, whose expression is not induced by dsRNA injection or viral infection, may be a good candidate to silence in order to study the involvement of bumblebee siRNA pathway upon viral infections in further studies. However, the possible association of Ago-2 to the miRNA pathway activity (Yang et al., 2014) should be considered when the silencing of *Ago-2* is adopted to study the antiviral activity of the siRNA pathway. 3) together with the absence of a 22 nt-long peak for SBPV, it seems that the siRNA pathway may not be sufficient but other pathways could play more important roles in the control of SBPV. It should also be noticed that artificial delivery of IAPV specific dsRNA (Hunter et al., 2010; Maori et al., 2009) or siRNA (Chen et al., 2014) has some rescue effect in honeybees, which is likely because of getting a plethora of virus specific dsRNA or siRNA to overshadow the effect of viral counterdefense. In addition, ingestion of IAPV specific dsRNA could also significantly reduce viral titers in bumblebees (unpublished data).

At the level of whole body, systemic immunity requires the coordination of various antiviral pathways. For instance, the cross-talk between RNAi and Jak/STAT pathway upon virus infection is mediated by Vago in a Dicer-2 dependent manner in mosquito (Paradkar et al., 2012). Although some evidence show the expression of *Vago* in honeybee is altered under DWV infection (Ryabov et al., 2014), its role in crosstalk is still unknown. It could be that virus defense by other pathways is triggered through Dicer-2 processing activity, and these pathways are more effective in comparison with the siRNA pathway to combat virus. In addition, the insect innate immune response is triggered with recognition of viruses in the form of PAMP or stress caused by viruses (Buchon et al., 2014; Lemaitre and Hoffmann, 2007; Wang et al., 2015). Different cellular pathways beyond the siRNA pathway are known to be triggered and they are possibly involved in virus defense. For instance, Toll, Imd, Jak/STAT, and Jnk, direct

involvement of these pathways could compensate the deficiency of the siRNA pathway to some extent. Intriguingly, aside from genes of the RNAi pathway (such as Dicer-like, Ago-2), *Toll-6* of the Toll pathway is also up-regulated in IAPV infected honeybees (Galbraith et al., 2015).

5. Conclusion

Our work provides different evidences to look at the possible involvement of bumblebee siRNA pathway upon viral infections, more specifically on possible Dicer-2 mediated antiviral activities in the bumblebee upon systemic infection of two different bee viruses. Both viruses replicate fast, and the host can cope with SBPV but not IAPV. The induction of *Dicer-2* expression by viruses or dsRNA, suggests the increased *Dicer-2* transcripts related with virus or dsRNA associated stresses. The predominant peak of ~22 nt-long vsiRNAs of IAPV may suggest the processing activity of Dicer-2. Although pre-silencing of *Dicer-2* in bumblebees can significantly influence gene silencing efficiency, it did not influence the viruses' gcn. Further studies about the downstream activity of the siRNA pathway upon virus infection, especially, VSR in inhibition of antiviral activity of bee siRNA pathway, and cross-talk of the siRNA pathway with other immune pathways, would enhance our understanding of the antiviral activity of the siRNA pathway of non-model insects against different viruses.

Supplementary materials

Table S1: Description of primers used in this chapter

Figure S1. Gene silencing in bumblebees by RNAi

Figure S2: Standard curves for detecting IAPV and SBPV genome copy number

Figure S3: Mortality of bees after IAPV feeding

Table S1: Description of primers used in this chapter (to be continued)

Gene	Acronym	Forward primer (5'-3')	Reverse Primer (5'-3')	Amplification efficiency
Elongation factor-1 α	ELF1α	CCAGGCATGCTGGTAACATTGGGAAAACCT	TTTCACGGAGATGTTCTTTACGACCCTTTCATT	1.970
60S ribosomal protein	RPL23	GAACTTAGGAAAA	TCTCCCTTGTTA	1.985
TATA-binding protein	TBP	ACTGGAGCAAAAGTGAAGAAGA	ACATCACAGCTGCCTACCATATT	1.966
Polyubiquitin	UBI	GGTATTTGGATGCCAGTGATTT	ATGGGCATTTCTACCCCTTTTA	2.003
Peptidylprolyl isomerase A	PPIA	TCGTAATGGAGTTGAGGAGTGA	CTTGGCACATGAAGTTTGGAAT	1.988
		TAATACGACTCACTATAGG	TAATACGACTCACTATAGGGAAGGGAAAAT	
Peptidylprolyl isomerase A	PPIA (dsRNA synthesis)	GCACTGGTGGAAGGTCCATCTTGTTACCCTTC	GGTGATGATTAGAAACAGATTCCGA	
Vago	Vago (qPCR)	AACGCAATTC	AACGCTGAT	2.012
		TAATACGACTCACTATAGGGAGACCTAGTCC	TAATACGACTCACTATAGGGAGAGTACGTACG	
Vago	Vago (dsRNA synthesis)	CGGAAGTCGAGA	AATTACAAGATCAACT	
tyrosine-protein kinase JAK2	Hop (qPCR)	TGGCACAATGTGTCTCATCTT	GAGGTACACAACGAGGTCCAG	1.969
		TAATACGACTCACTATAGGGAGATGTCCTTT	TAATACGACTCACTATAGGGAGATGACTGTCCT	
tyrosine-protein kinase JAK2	Hop (dsRNA synthesis)	GTTTCTGCTCTGGA	TCAGAATCTTGGA	
		TGGTCAAAACATCAAGAACAACCA	GATCGGGGCCATACGAACAT	
Dicer-2	Dicer-2 (qPCR)			1.952
		TAATACGACTCACTATAGGGAGAGCGAAGG	TAATACGACTCACTATAGGGAGA	
Dicer-2	Dicer-2 (dsRNA synthesis)	TGTCACCAAATGT	GGGTGTGTAAAGGCCTGCAA	

Gene	Acronym	Forward primer (5'-3')	Reverse Primer (5'-3')	Amplification efficiency
Dicer-1	Dicer-1 (qPCR)	AGAGGAACAA ATTTCAAATGA CGA	CTGATTCACAC GCAGGGGAT	1.931
Dicer-1	Dicer-1 (dsRNA synthesis)	TAATACGACTC ACTATAGGGAG A TTGGTCAAGCG ACATGGCAA	TAATACGACTC ACTATAGGGAG A AATGAGCTTGT CTATGCTTCGT	
Sid transmembrane family member 1	Sid (qPCR)	CGAGCCCATCA ACGGTAGAA	CGAGCCAAATC ACAAACGGA	
Sid transmembrane family member 1	Sid (dsRNA synthesis)	TAATACGACTC ACTATAGGGAG A TCATGGCAAAT CCCCCTTGT	TAATACGACTC ACTATAGGGAG A TGGCACAGTTA TACCGCCTT	
Peptidylprolyl isomerase A	PPIA (dsRNA synthesis)	TAATACGACTC ACTATAGGGCA CTGGTGGAAGG TCCATCT	TAATACGACTC ACTATAGGGAA GGGAAAATGG TGATGATTAGA A	
Israeli acute paralysis virus	IAPV (amply partial genome)	CACCAATCACG GACCTCACA	ACAGTGTTAGC TGCAGGACA	
Israeli acute paralysis virus	IAPV (qPCR)	CCATGCCTGGC GATTCAC	CTGAATAATAC TGTGCGTATC	1.999
slow bee paralysis virus	SBPV (amply partial genome)	CCAGGATCGCA TCCCCTTAG	TGTTGTCTCCC ACCTCATGC	
slow bee paralysis virus	SBPV (qPCR)	TCCAAGAGCAA GTATGCGGG	AGCATCAAAGC TAATTGCGGA	1.999
slow bee paralysis virus	SBPV (dsRNA synthesis)	TAATACGACTC ACTATAGGGAG AAGACCAGCTG GAGTTACAGG	TAATACGACTC ACTATAGGGAG ATGTTGTCTCC CACCTCATGC	

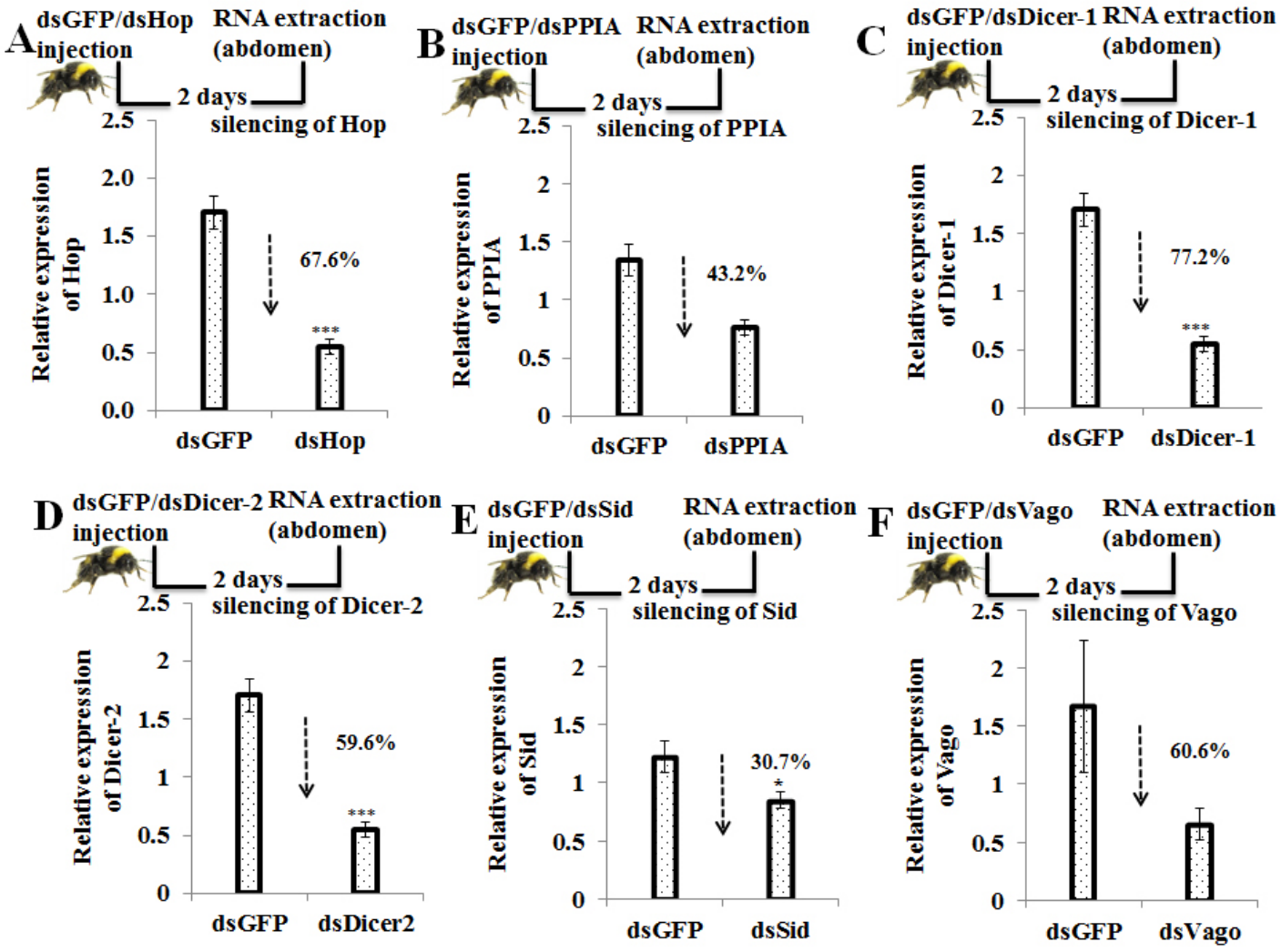


Figure S1. Gene silencing in bumblebees.
Hop (A), *PPIA* (B), *Dicer-1* (C), *Dicer-2* (D), *Sid* (E), *Vago* (F) in abdomen. After injection of 20 µg dsRNA in 20 µl per bee we detected a downregulation of 67.6% (T-test: t=7.462, df=12.706, *p<0.000*) of *Hop* (Figure 4A), 43.2 % (T-test: t=2.218, df=7, *p=0.062*) of *PPIA* (Figure 4B), 77.2% (T-test: t=-8.881, df=8, *p=0.000*) of *Dicer-1* (Figure 4C), 59.6% (T-test: t=7.958, df=24.160, *p<0.000*) of *Dicer-2* (Figure 4D), 30.7% (T-test: t=2.419, df=8, *p=0.042*) of *Sid* (Figure 4E), and 60.6% (T-test: t=1.997, df=6, *p=0.093*) of *Vago* (Figure 4F), respectively, compared with the controls of dsGFP injection.

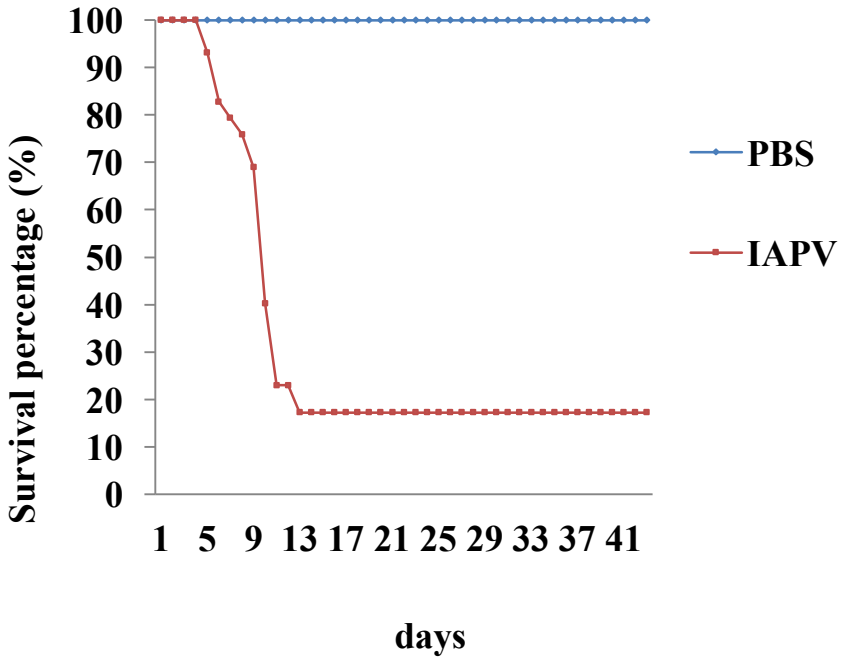


Figure S2: Mortality of bees after IAPV feeding

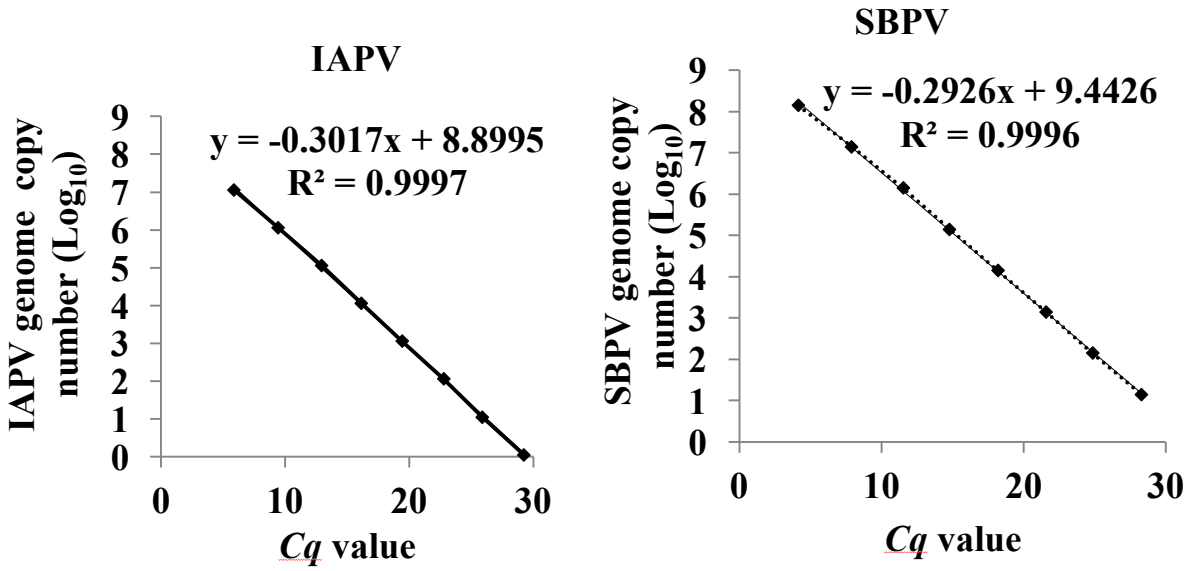


Figure S3: standard curves for detecting IAPV and SBPV genome copy number

Chapter III- Different expression of *Dicer-1*，*Ago-1*, and microRNAs upon different viral infections in *Bombus terrestris*

Parts of this chapter are submitted to:

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1. Introduction

The recent decline of bee populations including wild pollinators poses a threat to global food production (Goulson et al., 2015; Potts et al., 2010). Bee viruses are transmitted among different species, which has been particularly described between honeybees and bumblebees (Fürst et al., 2014; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010). The common bee viruses are mainly from families of *Dicistroviridae* and *Iflaviridae*, which are non-enveloped small icosahedral virions covering a positive sense single stranded RNA genome. Currently, viruses initially reported to infect the honeybees, such as ABPV, IAPV, KBV, DWV, BQCV, SBPV and SBV, are also being identified to infect bumblebees. Commercial colonies of honeybees and bumblebees are widely transported which could also disrupt local host-virus interactions in sympatric wild bees. Indeed, pathogen spillovers from managed colonies after transportation towards wild bees is regarded as a risk, particularly for endangered bee populations (see review (Meeus et al., 2011)).

As one of the major small non-coding RNAs, miRNA play an important role in regulating gene expression and influence various biological processes in eukaryotes. In insects, the miRNA pathway has been documented to be involved in different aspects of development, such as formation of germ cells, wing, and muscle, neurogenesis, apoptosis, and phenotypic plasticity (Asgari, 2013). The miRNA pathway is also well established to be involved in host-pathogen interactions (Asgari, 2013; Hussain and Asgari, 2014b). The canonical biogenesis of miRNA initiates in the nucleus where monocistronic, bicistronic or polycistronic transcripts are produced. These contain stem-loop structures known as the pri-miRNA. The pri-miRNA is cleaved by

Drosha and Pasha to liberate the pre-miRNA. After exportation to the cytoplasm, the pre-miRNA is cut by Dicer-1 to yield a miRNA duplex. The duplex strands are then sorted and the miRNA strand is loaded into RISC which typically includes Ago-1. Then, the mature miRNA binds to the target mRNA and leads to mRNA degradation or translational repression (Lucas and Raikhel, 2013). Besides, there are also non-canonical pathways of miRNA biogenesis, which are Drosha-independent but can be Dicer-dependent or Dicer-independent (Yang and Lai, 2011). The production and regulatory effects of miRNAs on insect-virus interactions could be complex. The first layer of complexity relates to the origin of miRNAs, which could be derived from the host or the virus. The second layer of complexity arises from the two-way interplay, meaning host encoded miRNAs can target genes from both host and viral genes and *vice versa* for virus encoded miRNAs (Asgari, 2015).

Differential expression of miRNAs has been associated with honeybee development and social behaviors (Behura and Whitfield, 2010; Greenberg et al., 2012; Liu et al., 2012b; Weaver et al., 2007). Recently, with the genome sequencing of two bumblebee species, *Bombus terrestris*, and *B. impatiens*, two datasets of miRNAs have been annotated for both species (Sadd et al., 2015). However, the regulatory effect of the miRNA pathway on bee-virus interaction is still unknown. Thus, in the current report, we comparatively analyzed the involvement of bumblebee miRNAs, in our experimental setup, upon infections of an avirulent virus SBPV and a virulent virus IAPV. First, we analyzed the expression of the core genes (*Dicer-1* and *Ago-1*) of the miRNA pathway upon virus infection; secondly, through small RNA sequencing, we analyzed the miRNA profiles following viral infections. To have a further insight into miRNA-mRNA interaction, we predicted the possible targets for these miRNAs. Finally, we silenced *Dicer-1* to analyze the outcome of SBPV infection. Our study may provide initial insights in the importance of miRNAs,

an additional layer in cell regulatory systems of bee-virus interactions.

2. Material and Methods

2.1. Insects and viral inoculation

The colonies of *B. terrestris* were obtained from Biobest NV (Westerlo, Belgium). The colonies used in this study were screened by RT-PCR (primers in supplementary table S1) to make sure that they were free of SBPV and IAPV. Newly emerged workers were collected and kept in micro-colonies fed with pollen and sugar water *ad libitum* for further experiments. All the micro-colonies were maintained in an incubator (Panasonic, Japan) at 29-31°C, 60-65% relative humidity, and continuous darkness.

Viruses inocula were produced as described previously (Niu et al., 2014a). The virus particles were counted by using transmission electron microscopy. To inoculate bees we injected an amount of 20,000 particles and 20 particles for SBPV and IAPV into five to eight days old workers in the side of the soft white-like cuticle between the 1st and 2nd abdominal segments by the nanoinjector (Eppendorf, Germany). PBS injected bumblebees served as control, which was the same solution used to purify viral samples. Although both viruses replicate very fast, we define SBPV as an avirulent virus infection pattern while IAPV represent a highly virulent infection pattern as the injection of SBPV causes no mortality and IAPV causes 100% mortality around 8 days even both viruses replicate very fast.

2.2. RNA isolation, cDNA synthesis, and qPCR

A total of 1.5~2 ml RLT buffer was initially used to homogenize the bumblebee by mortar and the supernatant was centrifuged three times to remove debris. Thereafter the protocol of the RNeasy mini kit (Qiagen, Germany) was followed. The TURBO DNA-free™ kit (Ambion, USA) was used to remove the possible genomic DNA contamination in RNA samples. The quantity and quality of RNA samples were checked by Nanodrop and electrophoresis on 1.5% agarose gel. An amount of 2 μg total RNA was used to synthesize the cDNA by SuperScript® II Reverse Transcriptase (Invitrogen, USA) using oligo (dT) primers. To make sure there was no genomic DNA contamination we checked cDNA samples by PCR with exon spanning primers for RPL23 (supplementary data table S1). The cDNA should produce an amplicon of 143 bp whereas the presence of genomic DNA will produce an extra amplicon of 452 bp. The qPCR was performed on a CFX96™ Real-Time PCR Detection with GoTaq® qPCR master (Promega, USA). Each reaction was performed in duplicate. The amplification specificities of primers used in this study were checked by both electrophoresis of the RT-PCR products and analysis of the dissociation curve by qPCR. A 10 fold serial dilution of cDNA was applied to calculate the amplification efficiency (supplementary data table S1). In addition, the RT-PCR products were sequenced in order to confirm their primers' amplification specificities.

2.3. Core gene expression of the miRNA pathway

We collected RNA samples at 8 h, 1, 2, 3, 7 and 13 days post injection (dpi) for SBPV and 8 h, 1, 2, and 3 dpi for IAPV, to analyze gene expressions. These samples were the same samples used in Chapter II (Section 2.5). For each biological replicate, the whole body of each individual bee was used to extract RNA. PBS injected bees were also collected in all different time points to serve as non-infected controls. Four to five biological replicates were included in each time point

for virus and PBS. The relative expression of key components, *Dicer-1* and *Ago-1*, of the miRNA pathway were normalized by internal control *PPIA* as the most stable reference gene during viral infections and different tissues (Niu et al., 2014a). The fold change of gene expression at each time point was given as the ratio of the relative gene expressions of the virus treated samples over the PBS controls collected at the same time point.

2.4. Small RNA sequencing and targets prediction of miRNAs

Small RNA sequencing was performed on RNA samples of SBPV and IAPV at 2 dpi. The PBS injected bees were included as control. Those samples were same ones used in Chapter II (section 2.7). Concentration and quality of the total extracted RNA was checked using the Quant-iT™ RiboGreen® RNA assay kit (Invitrogen, USA) and the RNA 6000 pico chip (Agilent Technologies, USA). Subsequently, 1 µg of total RNA was used to start the library preparation using the TailorMix miRNA Sample Preparation Kit v7 (SeqMatic, USA). Library preparation was carried out according to the manufacturer's instructions. The tRNA was added as carrier to minimize the loss of RNA via tube interaction. Libraries were quantified by qPCR, according to Illumina's protocol 'Sequencing Library qPCR Quantification protocol guide' (version February 2011). A high sensitivity DNA chip (Agilent Technologies, USA) was used to check the libraries' size distribution and quality. Single-end index 50 bp sequencing was performed on an Illumina MiSeq sequencer by loading 7 pM of each sample on the flowcell. A 10% PhiX spike-in was added as control. Four biological replicates for each treatment were sequenced by the NXTGNT sequencing platform from the Ghent University.

Ambiguous and low quality bases and adaptor sequences were trimmed from the sequencing reads using CLC Genomics Workbench 7.0.2. No ambiguous bases were allowed and a quality setting of 0.05 was applied. Reads smaller than 15 bp after trimming or reads containing more

than 10% of bases with Phred quality score lower than 20 were filtered with CLC Genomics Workbench 7.0.2 and fastX-toolkit 0.0.13.1, respectively. MiRNA reads were counted by CLC Genomics Workbench 7.0.2 based on annotated bumblebee miRNA dataset (Sadd et al., 2015). Differential expression analysis between virus-infected and non-infected bees was performed in the R Bioconductor-package limma on quantile normalized data. The results were corrected by multiple testing using a Benjamini-Hochberg False Discovery Ratio at a cut-off value of 0.05. To select differentially expressed miRNAs, we used a strict criteria based on miRNA counts (higher that 100), fold change (higher that 20% difference), and adjusted *p* value (less than 0.01).

Next, we predicted the potential targets of miRNAs to bumblebee mRNAs by RNA22 (Miranda et al., 2006). To further view the potential targets of miRNAs with differential expressions upon viral infections, we built a network of enrichment of Gene Ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by ClueGO (Bindea et al., 2009). Briefly, ClueGO is a Cytoscape plug-in App to facilitate the visualization of functionally grouped terms in the form of networks and charts. We used a maximum of 25 predicted targets per miRNAs (folding energy cut-off: -20 Kcal/mol) as input. The GO term retrieved from Uniprot (ftp://ftp.ebi.ac.uk/pub/databases/GO/goa/UNIPROT/) and KEGG annotation (http://www.genome.jp/kegg/kegg2.html) of *B. terrestris* were input with ClueGO main id, Entrez gene ID. The default parameters of ClueGO 2.1.7 was followed for network construction. In details, the GO term level was defined with minimum 3 and maximum 8. For each term cluster, it required a minimum of 3 genes with a minimum of 4.0 % clustered genes over the total genes in the term. The enrichment of predicted target genes of miRNAs to GO term/KEGG pathway was tested based on the hypergeometric distribution. A further multiple testing was followed by Bonferroni step-down. Finally, ClueGO created a binary gene-term matrix with the

selected terms and their associated genes. Based on this matrix, a term-term similarity matix was calculated using chance corrected kappa statistics to determine the association strength between the terms. In current analysis, we used a cut-off value 4.0 for kappa score, to represent the most significant (leading) terms in associated functional groups, which could be visualized in the network.

In addition, we also predicted the potential targets of *B. terrestris* miRNAs on SBPV and IAPV RNA (genome) by RNA22 (Miranda et al., 2006) and RNAhybrid (Krüger and Rehmsmeier, 2006), which used different prediction assumptions and thus made our prediction more reliable.

2.5. Validation of stability and differential expressions of miRNAs by RT-qPCR

The same samples collected at 8, 24, 48, 72 h (n=3) in section 2.3 for both virus injections and PBS controls were used to further analyze the expressions of miRNAs. An amount of 2 μg total RNA was used to prepare cDNA by miScript® II RT kit (QIAGEN, Germany). To quantify mature miRNAs in the study, the 5x miScript HiSpec buffer was used. The total volume of 20 μl reaction system was mixed by adding buffer, nucleics mix, reverse transcriptase mix, RNA template and RNase-free water, and then followed the manufacture' instruction. For RT-qPCR, an volume of 20 μl reaction system was mixed by adding SYBR green PCR master mix, universal primer (provided by the kit), microRNA specific primer (supplementary data table S2), template cDNA prepared above and RNase free water, by following the protocol of miScript SYBR® Green PCR kit (QIAGEN, Germany). Each reaction contained two technical replicates. To check the efficiency and specificity of miRNA primers, we evaluated the miRNAs' amplification efficiencies by using a 10 fold serial dilution of mixture cDNA and the melting curves in all tested cDNA samples.

The validation of references as the same principle of mRNA expressions through RT-qPCR, was also suggested before the detection of miRNAs' expressions (Kagias et al., 2014; Luo et al., 2014). To select good candidates reference miRNAs, we selected those with high counts (>100) and low expression variability indicated by sequencing data. Some miRNAs with significant changes upon viral infections revealed by the small RNA sequencing (section 2.4) were chosen to analyze their expressions in samples described above. The analysis of stable reference miRNAs were calculated by plug-in software geNorm in qBase^{PLUS} and the relative expressions of miRNAs were normalized by selected optimal reference miRNAs through qBase^{PLUS}.

2.6. Silencing *Dicer-1* and detection of its effect on viral genome copy number

A fragment of *Dicer-1* was amplified by PCR with target gene sequence specific primers plus T7 promoters (supplementary data table S1). This partial DNA template was purified by E.Z.N.A.® Cycle-Pure Kit (Omega, USA), and was sequenced in order to confirm the identity of amplification. Then, one microgram of template was used to synthesize dsRNA according to the guideline of MEGAscript® RNAi Kit (Invitrogen, USA). The concentration and quality of dsRNA were verified by Nanodrop and electrophoresis on 1.5% agarose gel. A total of 20 µg (20 µl) of dsRNA was injected into five to eight days old workers, and the same dose of dsGFP served as negative control. The abdomen of each individual was collected for RNA isolation. We used RNAi approach to investigate whether silencing of *Dicer-1* influences the genome copy number (gcn) of SBPV. First we silenced *Dicer-1* by injection of dsDicer1 (dsGFP serves as a control). After two days, the second injections were performed to inoculate bees by SBPV. Subsequently, the samples were collected for RNA extractions.

The measurement of SBPV gcn was based on a standard curve made from the Cq values (x) detected from a dilution of DNA templates of partial SBPV genome converting to corresponding gcn (y). In detail, a part of the SBPV genome (supplementary data table S1) was amplified and purified by E.Z.N.A.® Cycle-Pure Kit (Omega, USA). The partial sequence was confirmed by Sanger sequencing (LGC genomics, Germany). The concentration of purified templates was measured by Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, USA). The concentration was converted to gcn per µl by the online tool (URL: http://cels.uri.edu/gsc/cndna.html; Accessed date: 20/June/2014). A 10 fold serial dilution of templates was made to obtain a standard curve for each virus by qPCR. The equation is $y = -0.2926x + 9.4426$ ($R^2 = 0.9996$). The normalized gcn of each sample was represented by the ratio of the gcn calculated based on the standard curve and the normalization factor from the internal reference gene *PPIA* (Niu et al., 2014a) with the framework of qBase (Hellemans et al., 2007).

3. Results and Discussion

3.1. Significant effects of SBPV and IAPV infections on the expressions of *Dicer-1* and *Ago-1*

Aside from some non-canonical pathways of miRNA biogenesis, both *Dicer-1* and *Ago-1* are core components of miRNA biogenesis and determine the pathway's activity on gene regulation (Yang and Lai, 2011). In this study, we measured the expressions of *B. terrestris Dicer-1* and *Ago-1* upon two viral infections. Our results showed only a non-significant upregulation of both *Dicer-1* and *Ago-1* at 3 dpi of IAPV (Figure 1). While both *Dicer-1* (T-test: t=5.861, df=8, $p<0.001$) and *Ago-1* (T-test: t=5.370, df=4.578, $p=0.004$) were significantly upregulated at 2 dpi

of SBPV (Figure 1). In contrast, we detected a significant downregulation of *Ago-1* after 8 h injection of IAPV (T-test: t=2.616, df=6, *p=0.040*) (Figure 1B). Differential expression of *Dicer-1* and *Ago-1* may affect the activity of miRNAs. For instance, a significant upregulation of *Dicer-1* and/or *Ago-1* during desert locust phase transition and feeding was observed (Wynant et al., 2015). The expression of *Dicer* in human tumors can be either high or low (Huang et al., 2014a). Upon infection of non-human model (*Rhesus macaques*) by simian immunodeficiency virus, the protein level of Dicer increased in intestinal mucosa cells (Gaulke et al., 2014). On the other hand, upon viral infections, such as influenza A virus (Matskevich and Moelling, 2007) and vaccinia virus (Grinberg et al., 2012), the expression of *Dicer-1* was downregulated, which could be explained as a virus strategy to influence miRNAs biogenesis.

Regulation of miRNAs and their influence on host-virus interactions is complex (Asgari, 2015). For example, it is difficult to predict whether the increase or decrease in the expression of important genes in the miRNA biogenesis work in favor of the host or the virus. However, since we know the outcome of these two viral infections in bumblebees in our setup, we may speculate on who profits from the observed differential expressions of those genes. Both viruses replicate fast, but the two viruses differ largely in causing mortality in bees, SBPV as an avirulent virus and IAPV as an extremely virulent virus. Thus, the increase in *Dicer-1* and *Ago-1* expressions after SBPV infection may be needed for the host to try to control the virus infection. The downregulation of *Ago-1* at early stage of IAPV infection may be a strategy by IAPV in an attempt to facilitate its own replication and spreading. Intriguingly, with feeding solution containing IAPV, honeybees can exhibit neurological symptoms within 20-24 h, which leads to 753 differentially expressed genes in the fat body (Galbraith et al., 2015). This might indicate a very early interaction between the bees and IAPV.

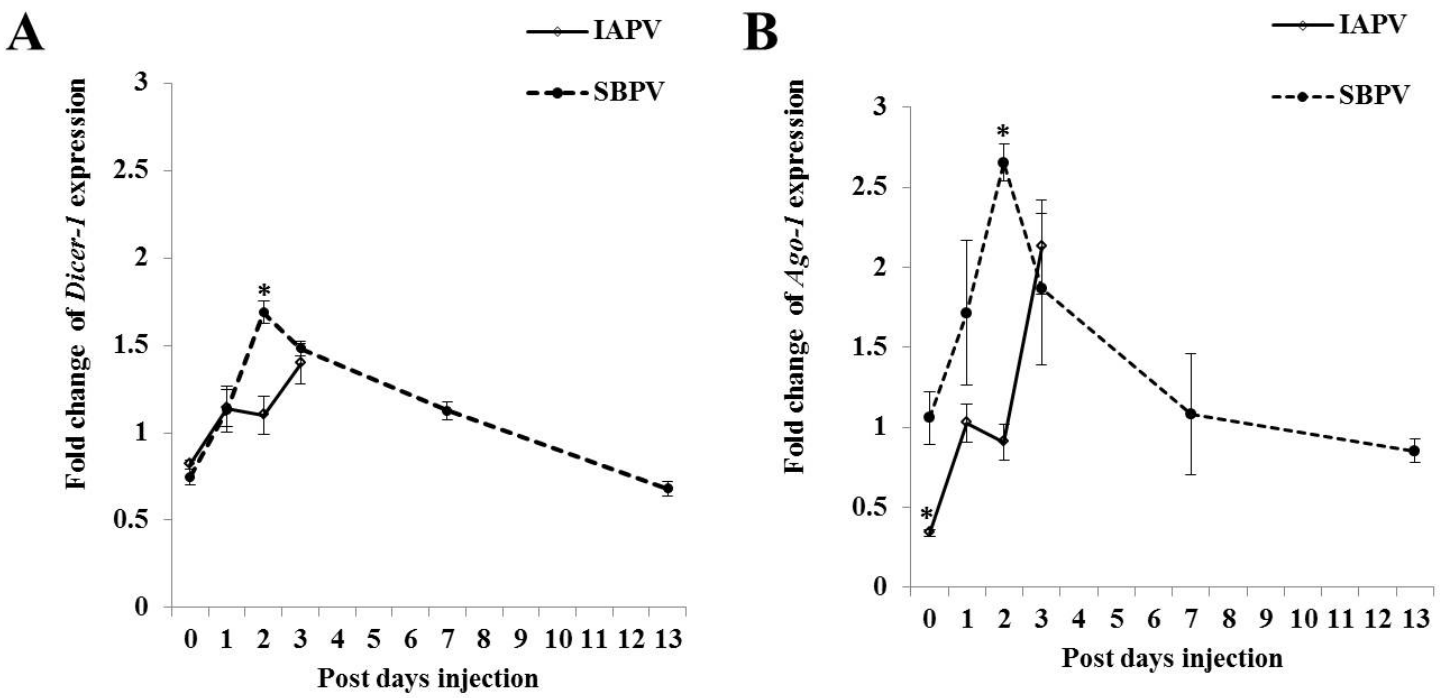


Figure 1: Fold changes of two core gene expressions in the miRNA pathway upon IAPV and SBPV infection.

(A): fold change of *Dicer-1* expression upon viral infections; (B): fold change of *Ago-1* expression upon viral infections. The fold changes of gene expression were equal to the ratio of the relative expression of each gene in virus infected samples over the relative expression of this gene in control samples (PBS injected bees). The relative expression of each gene was calculated based on internal reference gene *PPIA*. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p< 0.05$ was labelled with asterisks (*).

3.2. Small RNA sequencing reveals differentially expressed miRNAs upon SBPV and IAPV infections

Above we presented indications that the expression of components of the miRNA pathway could be altered upon viral infections in *B. terrestris* implying their potential involvement in host-virus interaction. To explore this possibility further, we used small RNA sequencing to find out which miRNA could be influenced. Currently, there are 217 miRNAs described for *A. mellifera* in miRBase, while in the two bumblebee species, *B. terrestris* and *B. impatiens*, 130 and 115 miRNAs have been identified, respectively (Sadd et al., 2015). Our results revealed that 17 and 12 miRNAs were differentially expressed upon infection with SBPV and IAPV, respectively (Table 1). From the 17 miRNAs differentially expressed in bees infected with SBPV, 10 miRNAs were upreguled and 7 were downregulated. In bees infected with IAPV, 12 miRNAs were differentially expressed, which included 7 upregulated and 5 downregulated miRNAs. Among all these differentially expressed miRNAs, 3 miRNAs (bte-miR-277, bte-bantam, and bte-miR-263a) were upregulated and 3 (bte-miR-3759, bte-miR-11, and bte-miR-24) were downregulated in both viral infections. Table 1 summarized the potential functions of the differentially abundant miRNAs based on the function of homologous miRNAs demonstrated in others insects. Two reappearing functions (apoptosis and energy homeostasis), known to be regulated by miRNAs, struck our attention as they have also been described to be important factors in virus-host interactions.

Apoptosis: Apoptosis or programmed cell death is an evolutionary conserved process in which the cells activate intracellular death pathways to terminate themselves in a programmed way in response to a wide variety of stimuli. It is clear that apoptosis in insects is an effective antiviral response to determine the outcome of DNA (Clem, 2005) and RNA viruses (Olagnier et al.,

2014). In view of host-virus interaction, apoptosis is one of the most ancestral defense mechanisms against virus infection; conversely, viruses may also interfere with the mechanism (Huang et al., 2014b; Urbanowski and Hobman, 2013) or take advantage of the stimulated apoptosis (Galluzzi et al., 2008). Homologous miRNAs of bte-miR-263a, bte-bantam, and bte-miR-11, three differentially expressed miRNAs during SBPV and IAPV infections, have been shown to be involved in apoptosis (Brennecke et al., 2003; Hilgers et al., 2010; Truscott et al., 2011). In addition, a homolog of bte-miR-263b, which is upregulated by SBPV infection but not IAPV, has been linked with apoptosis (Hilgers et al., 2010).

Energy homeostasis (insulin associated): Energy homeostasis plays a crucial role in host-virus interaction since viruses need energy in the form of ATP to replicate while hosts need energy to mount an immune response. The insulin signal transduction pathway responds to the nutritional status of the animal to control circulating sugar levels and fat metabolism. In addition, it can also influence the antiviral activity (Xu et al., 2013). Intriguingly, a homologue of bte-miR-278, upregulated by IAPV infection, is involved in energy homeostasis resulting in the regulation of levels of insulin (Teleman and Cohen, 2006) and regulation of the detoxification enzyme P450s (Lei et al., 2015). Homologs of bte-miR-13a upregulated by IAPV infection and bte-miR-13b upregulated by SBPV, regulate the expression of juvenile hormone (JH) (Lozano et al., 2015). JH plays an import role in regulation of insect growth and molting, which are also associated with ecdysone and insulin signaling pathways (Mirth et al., 2014). The homolog of another miRNA regulated by IAPV infection, bte-miR-305, was shown to regulate Notch and insulin pathways in the intestinal stem cells of the *Drosophila* gut (Foronda et al., 2014).

Intriguingly, a homolog of bte-miR-252a downregulated by SBPV, was shown to have a direct interaction with dengue virus (DENV) via targeting the DENV E protein transmembrane region

Table 1: Differentially expressed miRNAs upon virus infection

	Fold change		Targets of homologous miRNA in insects and its relative prediction in *Bombus terrestris*	
miRNA name	SBPV /PBS	IAPV /PBS	Homologous miRNA functions (target) in insects	Prediction of homologous targets or related targets in *Bombus terrestris*
bte-mir-13b	2.26		Juvenile hormone signaling pathway (Lozano et al., 2015)	insulin-like growth factor 2 mRNA-binding protein 1-like (LOC100647990); ecdysone-induced protein 75B, isoforms C/D-like, (LOC100644185)
bte-mir-927a	1.75			
bte-mir-277	1.60	1.45	Branched-chain amino acid catabolism (Esslinger et al., 2013) Neurodegeneration (Tan et al., 2012)	insulin receptor substrate 1-like (LOC100644779); insulin-degrading enzyme-like (LOC100644699)
bte-mir-263b	1.54		Apoptosis (Hilgers et al., 2010)	apoptosis-inducing factor 1, mitochondrial-like (LOC100651200); cell division cycle and apoptosis regulator protein 1-like (LOC100651179); apoptosis 2 inhibitor-like (LOC100647685); apoptosis regulator R1-like (LOC100649633); PRKC apoptosis WT1 regulator protein-like, transcript variant 1 (LOC100649375)
bte-bantam	1.49	1.28	Apoptosis (Brennecke et al., 2003); Stem cell regulation (Yang et al., 2009); Cell proliferation (Becam et al., 2011); Tumor suppressor (Zhang and Lai, 2013)	apoptosis 2 inhibitor-like (LOC100650673); SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1-like (LOC100645044); disks large 1 tumor suppressor protein-like (LOC100643982); fat-like cadherin-related tumor suppressor homolog (LOC100650960); cadherin-related tumor suppressor-like (LOC100651028)
bte-mir-9a	1.36		Wing development (Biryukova et al., 2009) Transcription factor of senseless (Cassidy et al., 2013; Li et al., 2006);	neurogenic locus Notch protein-like (LOC100645932); strawberry notch-like (LOC100649094)
bte-mir-31a	1.32			
bte-mir-263a	1.26	1.42	Apoptosis (Hilgers et al., 2010)	apoptosis-inducing factor 1, mitochondrial-like (LOC100651200); cell division cycle and apoptosis regulator protein 1-like (LOC100651179); apoptosis-inducing factor 3-like, transcript variant 1 (LOC100649657)
bte-mir-10	1.26			
bte-mir-750	1.22			

to be continued

	Fold change		Targets of homologous miRNA in insects and its relative prediction in *Bombus terrestris*	
miRNA name	SBPV /PBS	IAPV /PBS	Homologous miRNA functions (target) in insects	Prediction of homologous targets or related targets in *Bombus terrestris*
bte-mir-305		1.30	Intestinal stem cells (Foronda et al., 2014)	insulin-like growth factor-binding protein complex acid labile subunit-like (LOC100651887); insulin-degrading enzyme-like (LOC100644699); insulin-like peptide receptor-like (LOC100645413); insulin-like receptor-like (LOC100650952); insulin-like growth factor-binding protein complex acid labile subunit-like (LOC100643961)
bte-mir-13a		1.30	Juvenile hormone signaling pathway (Lozano et al., 2015)	insulin-like growth factor-binding protein complex acid labile subunit-like (LOC100647673); insulin-like receptor-like (LOC100651348); ecdysone-induced protein 75B, isoforms C/D-like (LOC100644185)
bte-mir-278		1.28	Energy homeostasis (Teleman and Cohen, 2006); P450s (Lei et al., 2015);	insulin-like peptide receptor-like (LOC100645413); insulin gene enhancer protein ISL-1-like (LOC100644486); cytochrome P450 18a1-like (LOC100642897); cytochrome P450 49a1-like (LOC100646342); cytochrome P450 4C1-like (LOC100651255); cytochrome P450 4g15-like (LOC100652170); cytochrome P450 6a13-like (LOC100646677); cytochrome P450 6a2-like (LOC100646434), (LOC100647785), (LOC100651291), (LOC100647041); cytochrome P450 6k1-like (LOC100642816), (LOC100642936), (LOC100643678), (LOC100647803), (LOC100648391), (LOC100648995), (LOC100650427); cytochrome P450 9e2-like (LOC100647566), (LOC100648545), (LOC100649871), (LOC100649988)
bte-mir-375		1.22		
bte-mir-252a	0.78		Dengue virus replication (Yan et al., 2014)	3'UTR of SBPV
bte-mir-316	0.72			
bte-mir-276	0.67		Olfactory (Li et al., 2013)	putative odorant receptor 13a-like (LOC100647497); odorant receptor Or2-like (LOC100644343); putative odorant receptor 82a-like (LOC100646456); odorant receptor 47b-like (LOC100646577); putative odorant receptor 13a-like (LOC100646817); putative odorant receptor 63a-like (LOC100644217)
bte-mir-3718a	0.66			
bte-mir-3759	0.57	0.76		
bte-mir-11	0.56	0.61	Apoptosis (Truscott et al., 2011)	DNA damage-binding protein 1-like (LOC100650523)
bte-mc-24	0.44	0.68		
bte-mc-753		0.75		
bte-mir-283		0.71		

(Yan et al., 2014). Through RNAhybrid and RNA22, we also predicted that bte-miR-252a could possibly target the 3'UTR of SBPV (Table 2). Whether a similar mechanism of virus regulation is present in bumblebee remains to be explored.

3.3. *In silico* target prediction of differentially expressed miRNAs shows a possible host-virus interaction network mediated by miRNAs

Until now, the analyses of targets of miRNAs have only shown the tip of the iceberg of their potential in gene regulation. The current understanding is that one miRNA may target hundreds of genes, while a gene may be regulated by multiple miRNAs. Based on RNA22, we identified a total of 7465 genes possibly targeted by 130 miRNAs in *B. terrestris*. Among them, 7216 genes were possibly targeted by the 23 differentially expressed miRNAs upon virus infection. Some predicted targets of these 23 miRNAs were visualized in the GO and KEGG enrichments by ClueGO in two groups: 17 miRNAs differentially expressed by SBPV infection, and 12 miRNAs differentially expressed by IAPV infection. The analysis showed that there were 32 and 21 groups of GO/KEGG pathways that popped out in the network of SBPV (Figure 2) and IAPV (Figure 3). A number of 11 GO/KEGG pathways were common in the two datasets, which is consistent with the 6 common miRNAs influenced by SBPV and IAPV infections. Generally, these pathways can be associated with a certain level of molecular activities in the view of virus-host interaction, such as DNA/RNA, protein, metabolism, cell activity, host disease, and host antiviral immunity (Table 3). These visualizations of miRNA-mRNA-GO/KEGG enrichments may show a possible host-virus interaction network. Moreover, these potential targets reported of the homologous miRNAs also showed an overlap with our predictions (Table 1). It may be a good start to verify the role of apoptosis and insulin associated energy homeostatis in host-virus interaction based on miRNAs.

Table 2: Predicted targets of host miRNAs to viral genomes

Target	miRNA	MFE (Kcal/mol)	Binding Site Start Position	Target Gene Annotation
SBPV	bte-mir-276	-21.8	7261	Polyprotein_ORF
SBPV	bte-mir-276	-20.7	2285	Polyprotein_ORF
IAPV	bte-bantam	-25	4919	Polymerase Polyprotein_ORF
IAPV	bte-mc-24	-28.5	6219	Polymerase Polyprotein_ORF
IAPV	bte-mc-24	-28.1	6554	UTR
IAPV	bte-mc-24	-25	328	5'UTR
SBPV	bte-mir-252a	-29.6	9370	3'UTR
SBPV	bte-mir-9a	-25.1	3777	Polyprotein_ORF
IAPV	bte-mir-11	-21.8	7752	Structural Polyprotein_ORF
IAPV	bte-mir-283	-20.8	1145	Polymerase Polyprotein_ORF
IAPV	bte-mc-24	-22.5	9474	3'UTR
IAPV	bte-mc-753	-22.4	7878	Structural Polyprotein_ORF
SBPV	bte-mir-263b	-21.9	3705	Polyprotein_ORF
SBPV	bte-mir-276	-22.9	2500	Polyprotein_ORF
SBPV	bte-mir-3759	-23.3	7714	Polyprotein_ORF
SBPV	bte-mc-24	-20.1	9239	3'UTR
SBPV	bte-mc-753 *	-26.3	7748	Polyprotein_ORF
IAPV	bte-mir-263b*	-24.2	8501	Structural Polyprotein_ORF
IAPV	bte-mir-9a*	-20.2	5318	Polymerase Polyprotein_ORF
IAPV	bte-mir-252a*	-22.2	3819	Polymerase Polyprotein_ORF
IAPV	bte-mir-252a*	-21.8	7091	Structural Polyprotein_ORF
IAPV	bte-mir-263b*	-20.1	6255	Polymerase Polyprotein_ORF
IAPV	bte-mir-750*	-21.9	1993	Polymerase Polyprotein_ORF
SBPV	bte-mir-305*	-22.7	785	Polyprotein_ORF

* Not differentially expressed due to that particular viral infection

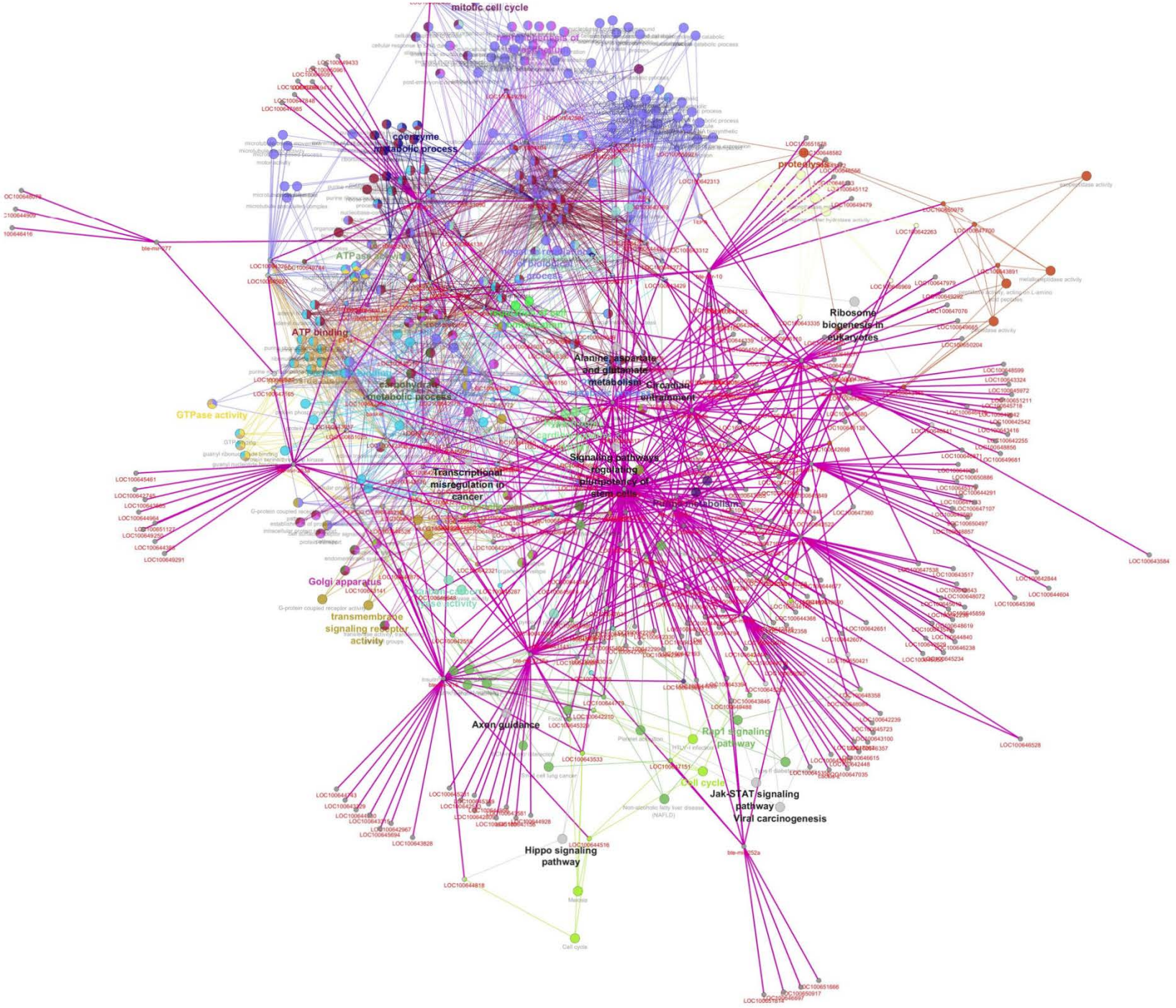


Figure 2: The enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of predicted targets of differentially expressed miRNAs by SBPV infection. For each miRNA, a maximum of 25 predicted targets (folding energy cut-off: -20 Kcal/mol) was used as input to ClueGO. The GO and KEGG presented in the figure were based on Kappa score higher than 0.4.

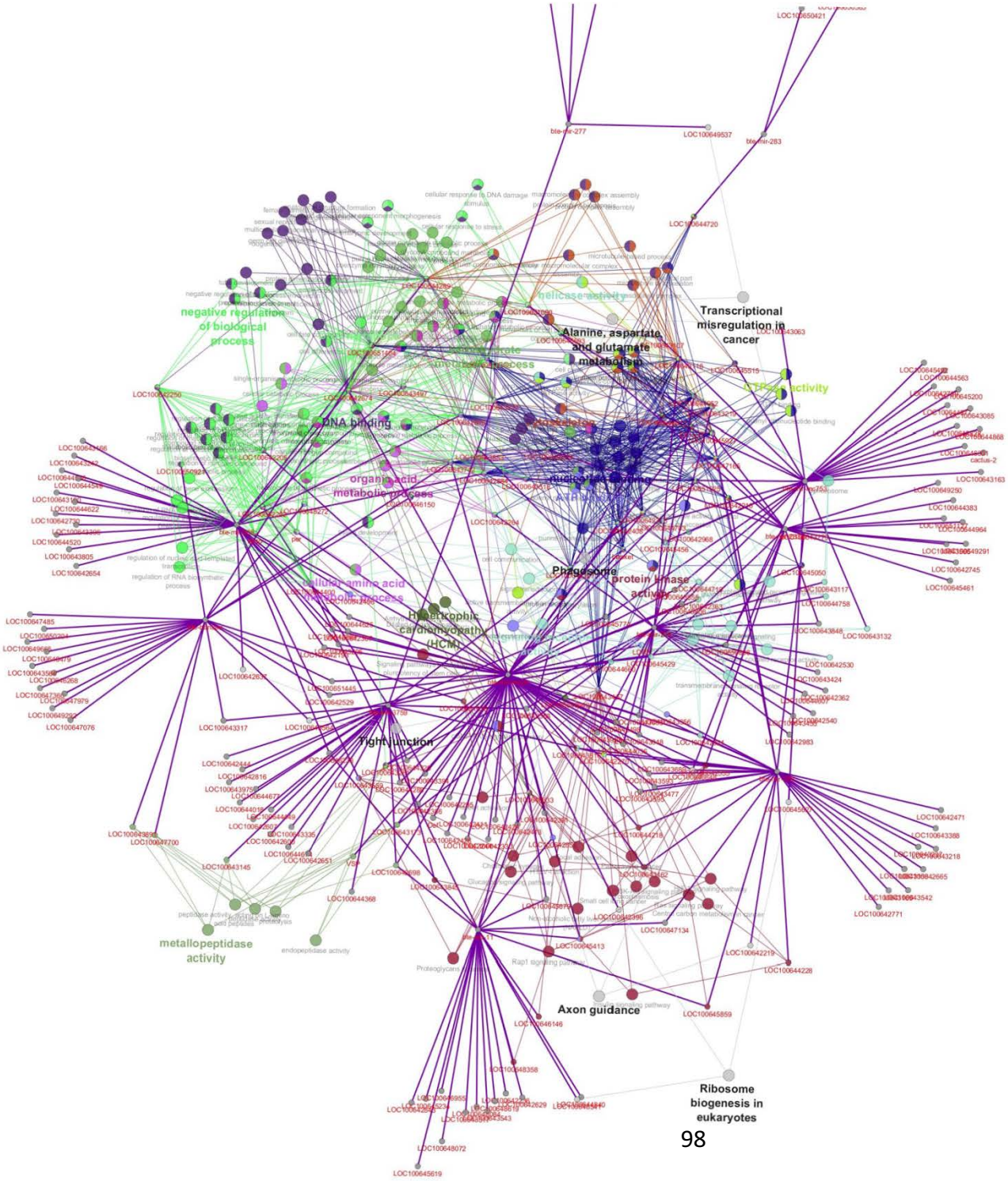


Figure 3: The enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of predicted targets of differentially expressed miRNAs by IAPV infection. For each miRNA, a maximum of 25 predicted targets (folding energy cut-off: -20 Kcal/mol) was used as input to ClueGO. The GO and KEGG presented in the figure were based on Kappa score higher than 0.4.

Table 3: The most significant enriched GO/KEGG from the targets of differentially expressed miRNA upon virus infection.

Differentially expressed miRNAs by SBPV		Differentially expressed miRNAs by IAPV	
GO/KEGG ID	Name	GO/KEGG ID	name
KEGG:00250	**Alanine, aspartate and glutamate metabolism**	**KEGG:00250**	**Alanine, aspartate and glutamate metabolism**
GO:0005524	**ATP binding**	**GO:0005524**	**ATP binding**
KEGG:04360	**Axon guidance**	**KEGG:04360**	**Axon guidance**
GO:0005975	**carbohydrate metabolic process**	**GO:0005975**	**carbohydrate metabolic process**
GO:0003924	**GTPase activity**	**GO:0003924**	**GTPase activity**
KEGG:05410	**Hypertrophic cardiomyopathy (HCM)**	**KEGG:05410**	**Hypertrophic cardiomyopathy (HCM)**
GO:0048519	**negative regulation of biological process**	**GO:0048519**	**negative regulation of biological process**
GO:0000166	**nucleotide binding**	**GO:0000166**	**nucleotide binding**
GO:0006082	**organic acid metabolic process**	**GO:0006082**	**organic acid metabolic process**
KEGG:03008	**Ribosome biogenesis in eukaryotes**	**KEGG:03008**	**Ribosome biogenesis in eukaryotes**
KEGG:05202	**Transcriptional misregulation in cancer**	**KEGG:05202**	**Transcriptional misregulation in cancer**
GO:0016887	ATPase activity	GO:0006520	cellular amino acid metabolic process
GO:0016830	carbon-carbon lyase activity	GO:0005856	cytoskeleton
KEGG:04110	Cell cycle	GO:0003677	DNA binding
KEGG:04713	Circadian entrainment	GO:0006259	DNA metabolic process
GO:0006732	coenzyme metabolic process	GO:0004386	helicase activity
GO:0005794	Golgi apparatus	GO:0008237	metallopeptidase activity
KEGG:04390	Hippo signaling pathway	KEGG:04145	Phagosome
GO:0016788	hydrolase activity, acting on ester bonds	GO:0004672	protein kinase activity
KEGG:04630	JAK/STAT signaling pathway	GO:0038023	signaling receptor activity
GO:0000278	mitotic cell cycle	KEGG:04530	Tight junction
GO:0002009	morphogenesis of an epithelium		
GO:0001882	nucleoside binding		
GO:0031090	organelle membrane		

to be continued

Differentially expressed miRNAs by SBPV		Differentially expressed miRNAs by IAPV	
GO/KEGG ID	Name	GO/KEGG ID	name
GO:0006508	Proteolysis		
KEGG:00230	Purine metabolism		
KEGG:04015	Rap1 signaling pathway		
GO:0010646	regulation of cell communication		
GO:0010033	response to organic substance		
KEGG:04550	Signaling pathways regulating pluripotency of stem cells		
GO:0004888	transmembrane signaling receptor activity		
KEGG:05203	Viral carcinogenesis		

Notes: pathways with bold are the same GO/KEGG appeared in both SBPV and IAPV dataset. These GO/KEGG could be associated with certain biological functions in the view of host-virus virus interaction. Network of SBPV influenced miRNAs-targets-GO/KEGG: In DNA/RNA associated GO/KEGG, (GO:0005524), (GO:0003924), (GO:0000166), (GO:0016887), (GO:0001882), (KEGG:00230). In protein associated GO/KEGG, (KEGG:00250), (KEGG:03008), (GO:0005794), (GO:0006508). In metabolism associated GO/KEGG, (GO:0005975), (GO:0006082), (GO:0016830), (GO:0006732). In cell activity associated GO/KEGG, (KEGG:04110), (GO:0000278), (KEGG:04015), (GO:0010646), (GO:0010033), (KEGG:04550), (GO:0004888). In host disease associated GO/KEGG, (KEGG:05410), (KEGG:05202), (KEGG:05203). In host antiviral immunity associated GO/KEGG, (KEGG:04390), (KEGG:04630). Network of IAPV influenced miRNAs-targets-GO/KEGG: In DNA/RNA associated GO/KEGG, (GO:0005524), (GO:0003924), (GO:0000166), (GO:0003677), (GO:0006259), (GO:0004386). In protein associated GO/KEGG, (KEGG:00250), (KEGG:03008), (GO:0006520), (GO:0008237), (GO:0004672). In metabolism associated GO/KEGG, (GO:0005975), (GO:0006082). In cell activity associated GO/KEGG, (GO:0005856), (GO:0038023), (KEGG:04530). In host disease associated GO/KEGG, (KEGG:05410), (KEGG:05202). In host antiviral immunity Phagosome (KEGG:04145).

In addition, we also explored if host miRNAs could directly target viral RNAs (genomes) using RNAhybrid and RNA22. The results identified a total of 24 target sites on SBPV and IAPV genomes by host miRNAs (Table 2). The predicted target sites were located in the UTRs as well as ORFs (open reading frame). Of these 24 targets sites, 16 were predicted to be targeted by differentially expressed miRNAs upon virus infections. We then applied a Chi square test to analyze whether these predictions acted just by chance. Chi square tests indicated that an significant difference of the number of differentially expressed bumblebee miRNAs upon each viral infection in targeting its own viral RNAs (genomes), and all bumblebee miRNAs in targeting viral RNAs (genomes) for both SBPV (χ^2=14.816, $p<0.001$) and IAPV (χ^2=13.422, $p<0.001$) analysis. This could suggest that the host miRNAs might also play a role in directly regulating SBPV/IAPV genome or RNA. Further analyses are needed to elucidate these direct miRNAs-virus interaction, which may give us another strategy to use miRNAs to control the viruses or in a combination with the strategy of virus specific siRNA and dsRNA through the siRNA pathways (Niu et al., 2014b).

3.4. Validation of stability and differentially expressed miRNAs by RT-qPCR

Until now, there are no stable reference miRNAs known to normalize the data upon viral infections and non-infection in bumblebees. With the pre-selection of more than 10 candidates in their performance based on primers efficiency and specificity (supplementary data Table S2 and Figure S1), we eventually validated six miRNAs, including bte-mir-14, bte-mir-100, bte-mir-34, bte-mir-87, bte-mir-184 and bte-mir-281, in the same samples used to validate several key differentially expressed miRNAs indicated by small RNA sequencing. By geNorm, the results showed that all these candidate reference showed an *M* value less than 1 except that bte-mir-184 showed an *M* value of 1.002 (supplementary data Figure S2A). Pairwise variations indicated a lowest *V* value of 0.165 (V4/5) upon the combination of the four most stable reference miRNAs (supplementary data Figure S2B). These results suggested the optimal references within these candidate references were the combination of four microRNAs, mir-281+bte-mir-34+bte-mir-87+bte-mir-100, for data analysis of miRNA expressions upon different injection time points (8, 24, 48, and 72 h) of IAPV and SBPV, respectively.

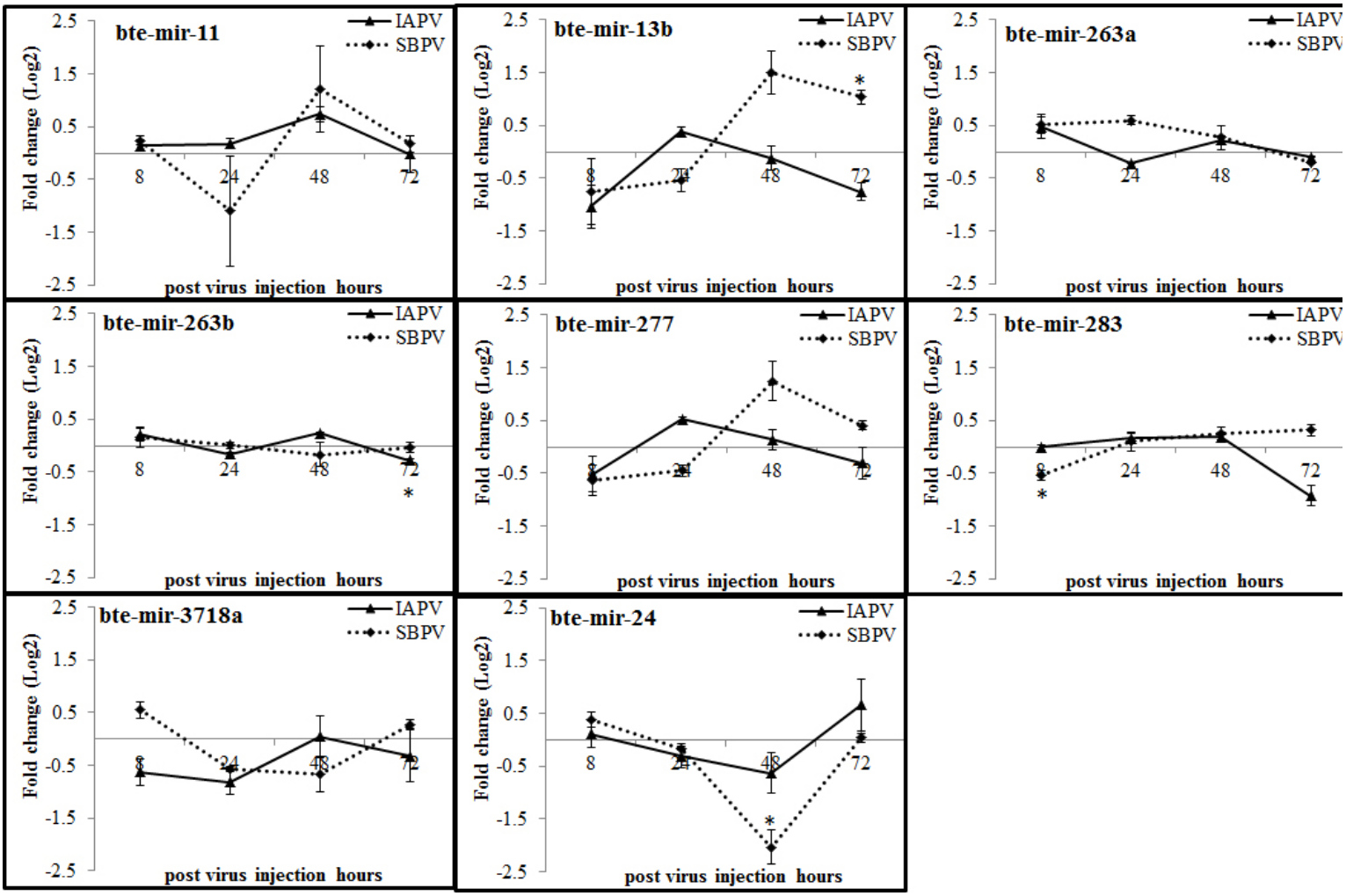


Figure 4: Fold changes of key differentially expressed microRNAs by sequencing upon different injection time points of IAPV and SBPV.

The fold changes of microRNA expression were equal to the ratio of the relative expression of microRNA in virus infected samples over the relative expression of this microRNA in control samples (PBS injected bees). The relative expression of microRNA was calculated based the combination of four optimal reference microRNAs: mir-281+bte-mir-34+bte-mir-87+bte-mir-100. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p<0.0125$ was labelled with asterisks (*).

To further analyze the differentially expressed miRNAs from sequencing, with the pre-selection based on primers efficiency and specificity (supplementary data Table S2 and Figure S1), we eventually evaluated the stabilities of bte-mir-11, bte-mir-13b, bte-mir-263a, bte-mir-263b, bte-mir-277, bte-mir-283, bte-mir-3718a and bte-mc-24, in all samples by RT-qPCR. The relative expressions of these miRNAs were normalized by four miRNAs selected above (mir-281+bte-mir-34+bte-mir-87+bte-mir-100). In each time points, we had separated PBS controls for two viruses, thus we used independent t-test to separate means but with bonferroni correction as four times multi-comparison for each virus at four time points between virus injections and PBS injections. Therefore, the strict *p* value of 0.0125 was used to judge the statistical difference of miRNAs expressions. In Figure 4, we calculated the fold change (log2 transformed) of miRNAs based on the division of miRNAs expression in virus injected samples and the very time points of PBS injected samples. We found that bte-mir-13b was upregulated at 48 and 72 h post injection of SBPV, but only with significant difference at 72 h ($p=0.010$). The significant downregulation of bte-mc-24 ($p<0.001$) was detected at 48 h post injection of SBPV and a tendency of downregulation at 48 h post injection of IAPV. These results were consistent with our miRNA sequencing results which showed these two miRNAs were the most changed miRNAs (Table 1). Other six miRNAs did not show the same results as the sequencing results at 48 h post viral injections. This may be associated with the fact that different samples were used in this section compared with the samples of miRNA sequencing, and virus titers were variable in each treatment among individuals. Bte-mir-263b was found to be significantly downregulated ($p=0.010$) at 72 h post injection of IAPV, and the significant downregulation of bte-mir-283 ($p=0.007$) was detected at 8 h post injection of SBPV. These results might suggest that some miRNAs could also play roles at early and late infectious stages.

3.5. Depletion of *Dicer-1* by RNAi did not lead to an altered genome copy number of SBPV

Although our results showed an altered expression of *Dicer-1* and *Ago-1* and differential abundance of miRNAs upon virus infections, the role of the miRNA pathway itself on the outcome of viral pathogenicity is not clear. Therefore, we used RNAi to silence *Dicer-1* to detect its influence on the outcome of SBPV gcn. Our results showed a ~77% depletion of *Dicer-1* transcripts after two days of injection of dsRNA (Figure 5A), but this pre-silencing of *Dicer-1* did not result in a significant difference in SBPV gcn (Figure 5B). Silencing or mutation of *Dicer-1* in insects can significantly inhibit the production of some host miRNAs (Lozano et al., 2015; Wang et al., 2013b) and viral miRNA-like small RNAs (Hussain and Asgari, 2014a), and the assembly of miRNA-RISC (Liu et al., 2007). However, why we did not find an effect on SBPV gcn after silencing *Dicer-1* in *B. terrestris*? One explanation could be that silencing of *Dicer-1* in *B. terrestris* might influence the benefits of both players in the interaction. For instance, some miRNAs could be of benefit to the host to recruit pathway with antiviral activitys or directly act on viral genome. In contrast, some miRNAs could be of benefit to the virus to replicate and spread which are normally host miRNAs manipulated by virus and/or viral encoded miRNAs.

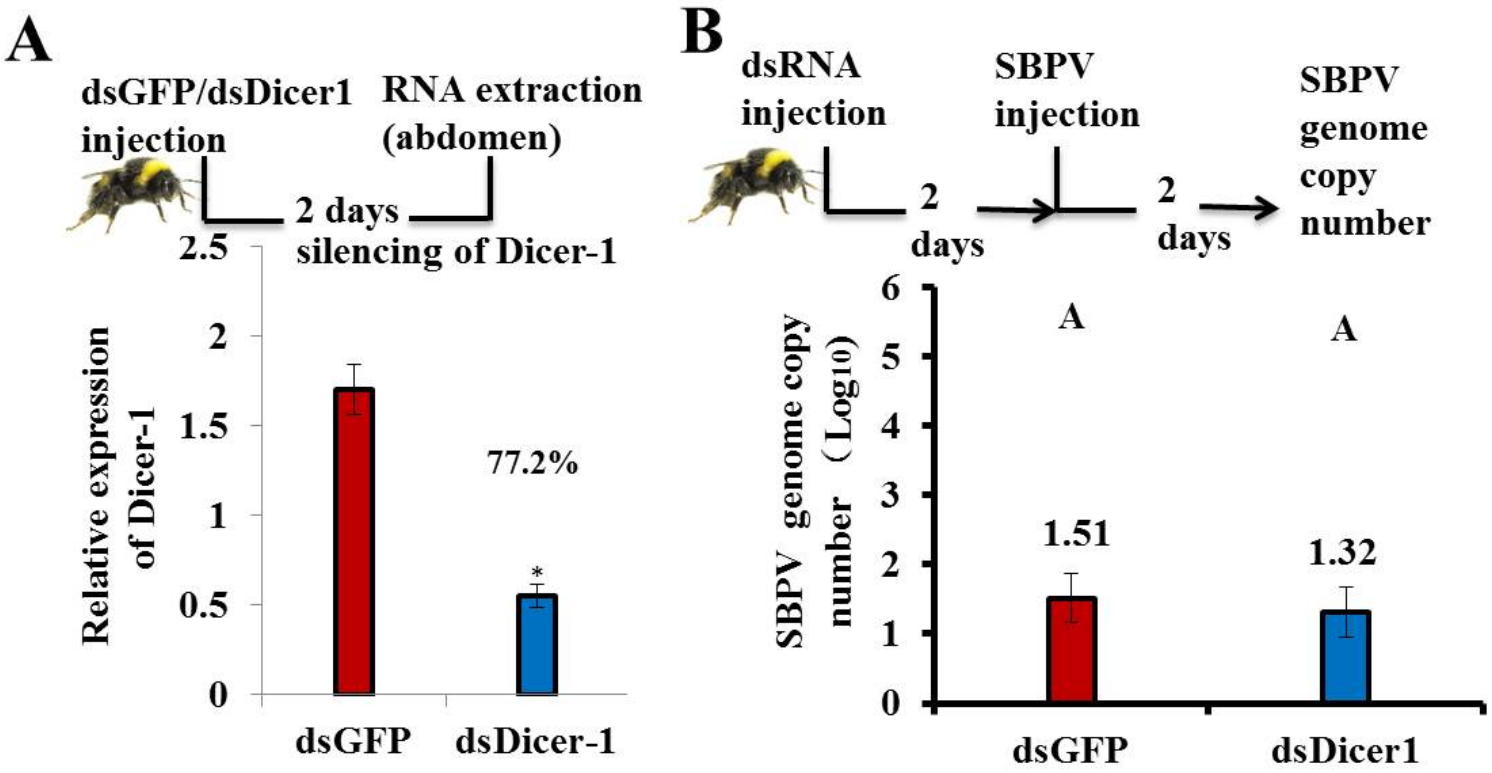


Figure 5: Silencing of *Dicer-1* and its effect on the genome copy number of SBPV.

(A): the injection of dsDicer-1 (n=5) to target *Dicer-1* led to 77.2% reduction of its transcript levels compared with injection of the negative control dsGFP (n=5). (B): pre-silencing of *Dicer-1* led to no difference of genome copy number of SBPV (n=12).

Thus, silencing *Dicer-1* would sabotage the activities of miRNAs benefited both the host and the virus, which might lead to an equivalent situation for both parties. Secondly, it could be that silencing of *Dicer-1* may not influence the miRNAs produced through non-canonical pathways (Asgari, 2015; Yang and Lai, 2011), which might play a more important role here. Nevertheless, silencing of *Dicer-1* may not lead to a complete inhibition of Dicer-1 activity, but a temporal silencing of *Dicer-1*, and perhaps does not turnover the activity of the whole pathway.

4. Conclusion

The miRNA pathway plays an important role in mediating insect host-virus interactions. In our experiments, we found that an avirulent virus infection induced by SBPV and a virulent infection induced by IAPV in bumblebee *B. terrestris* could alter the expression of the miRNA pathway core genes and abundance of miRNAs. It seems that based on the level of induction of *Dicer-1* and *Ago-1* and the number of differentially expressed miRNAs, the miRNA regulation was more disturbed or influenced after SBPV infection compared with IAPV infection. The potential target predictions and GO and KEGG enrichments were produced for initial visualization purposes and still need biological evidence, while it reflected the potential of bumblebee miRNAs could be involved in various aspects of molecular activities in the view of virus-host interaction. In addition, some differentially expressed miRNAs upon virus infection may directly target the viral genome based on our prediction. In summary, our study opens a new insight into bee-virus interaction meditated by the miRNA pathway, which might also enhance our general understanding in interactions between insects and various pathogenic viruses.

Supplementary data 1

Table S1: Primers used in this chapter

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')	Amplification efficiency (AE)	NCBI accession number	reference
PPIA （qPCR）	TCGTAATGGAGTTGAGGAGTGA	CTTGGCACATGAAGTTTGGAAT	1.988	XM_003402218.2	(Niu et al., 2014a)
RPL23 （qPCR）	GGGAAAACCTGAACTTAGGAAAA	ACCCTTTCATTTCTCCCTTGTTA	1.985	XM_003400707.2	(Niu et al., 2014a)
IAPV (amply partial genome)	CACCAATCACGGACCTCACA	ACAGTGTTAGCTGCAGGACA		EU436443.1	
IAPV (qPCR)	CCATGCCTGGCGATTCAC	CTGAATAATACTGTGCGTATC		EU436443.1	(de Miranda et al., 2010a)
SBPV (amply partial genome)	CCAGGATCGCATCCCCTTAG	TGTTGTCTCCCACCTCATGC		GU938761.1	
SBPV (qPCR)	TCCAAGAGCAAGTATGCGGG	AGCATCAAAGCTAATTGCGGA		GU938761.1	
Dicer-1 （qPCR）	AGAGGAACAAATTTCAAATGACGA	CTGATTCACACGCAGGGGAT	1.931	XM_003401907.2	
Dicer-1 （ dsRNA synthesis）	TAATACGACTCACTATAGGGAGATTGGTCAAGCGACATGGCAA	TAATACGACTCACTATAGGGAGAAATGAGCTTGTCTATGCTTCGT		XM_003401907.2	
Ago-1（qPCR）	AGTAGTGGGGTCTTTCTCGC	TGCTCGCACTTATTTTACAATGACA	2.069	XM_012315499.1	

Table S2: Specific primers to detect miRNA expression by RT-qPCR.

Goal	microRNA	specific primer (5'-3')	amplification efficiency
expression test	bte-mir-11	CATCACAGGCAGAGTTCTAGTT	2.063
	bte-mir-13b	TATCACAGCCATTTTTGACGATT	1.992
	bte-mir-263a	AATGGCACTGGAAGAATTCACG	2.046
	bte-mir-263b	CTTGGCACTGGAAGAATTCACAG	2.061
	bte-mir-277	TAAATGCACTATCTGGTACGACA	2.163
	bte-mir-283	AAATATCAGCTGGTAATTCTG	2.056
	bte-mir-3718a	TCCCCTGTCCTGTCCCGATAGT	2.015
	bte-mc-24	TGTGGGGCGGCGTCCGGGTCACT	2.092
Candidate reference	bte-mir-184	TGGACGGAGAACTGATAAGG	2.076
	bte-mir-14	GGGGGTGAGAAACTGGCTTGGCT	2.076
	bte-mir-100	AACCCGTAGATCCGAACTTGTG	2.011
	bte-mir-87	GTGAGCAAAGTTTCAGGTGTGT	2.009
	bte-mir-34	TGGCAGTGTTGTTAGCTGGTTGTG	2.017
	bte-mir-281	AAGAGAGCTATCCATCGACAGT	2.057

Figure S1: Melt curves of miRNA primers by RT-qPCR performed in all samples.

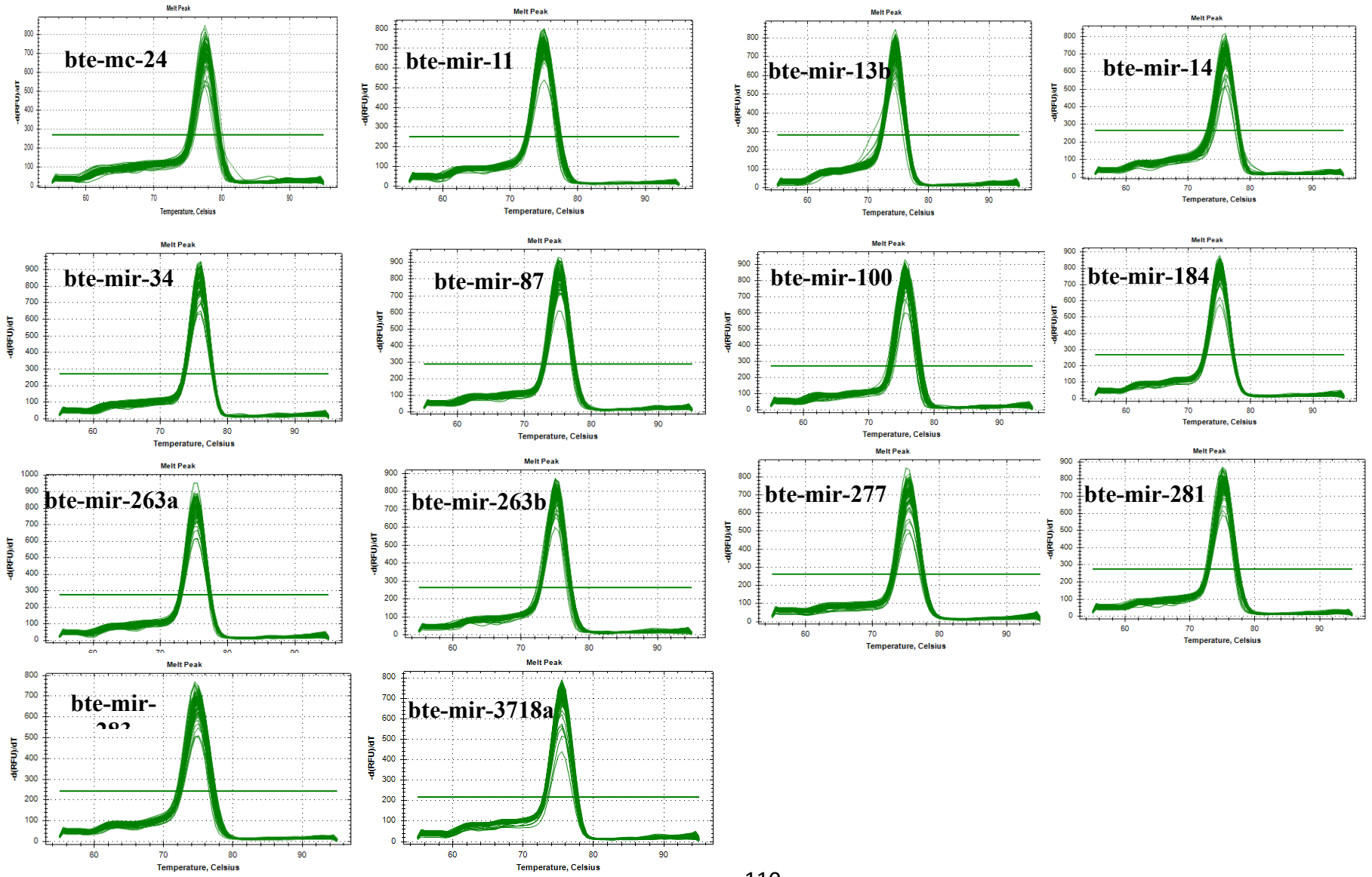
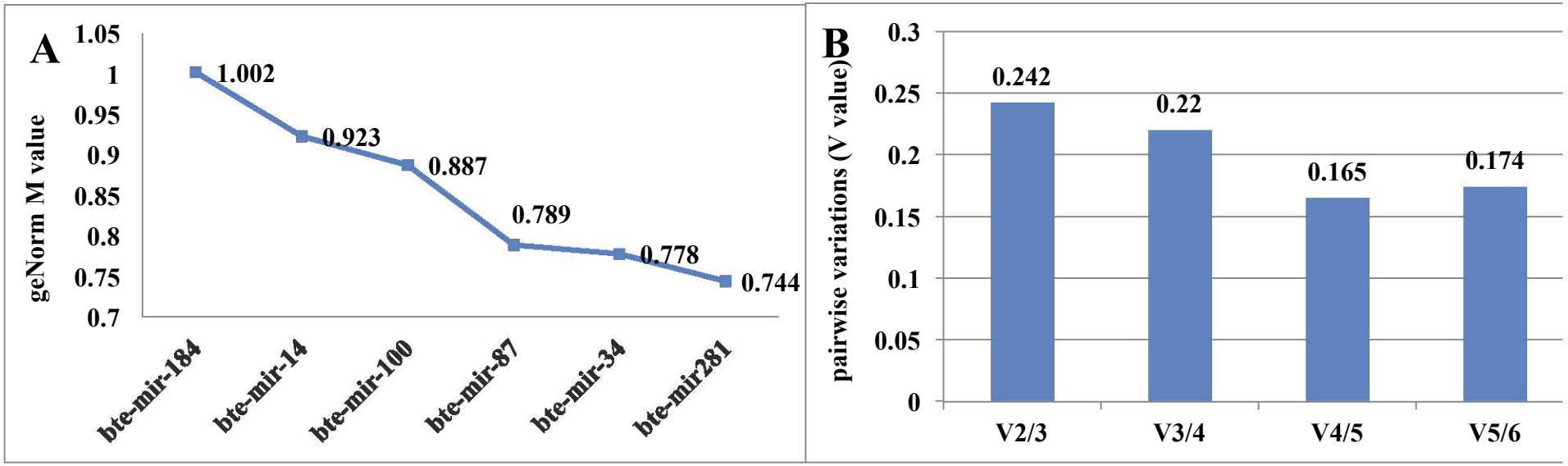


Figure S2: Validation of miRNA references for RT-qPCR in all samples.



Chapter IV-Involvement of *Bombus terrestris* JAK/STAT pathway in antiviral response and possible interaction with the small interfering RNA pathway through Vago

Parts of this chapter are submitted to:

Niu, J, Meeus I, Smagghe G, 2015. Involvement of *Bombus terrestris* JAK/STAT pathway in antiviral response and possible interaction with the small interfering RNA pathway through Vago. Scientific Reports submitted.

1. Introduction

Insects and other invertebrates lack adaptive immune system, which highlights that the innate immunity in these species is critical to modulate the outcome of virus infections. Currently, the antiviral innate immune pathways in insects can be grouped into two strategies of either nucleotides-based (such as RNAi) or protein-based (such as Toll, Imd and the JAK/STAT (Buchon et al., 2014; Merkling et al., 2013). Taken the siRNA pathway (sub-pathway of RNAi) as example for nucleotides-based antiviral strategy in insects: during viral infection, especially for RNA viruses, virus related dsRNAs are generated, from viral dsRNA replication intermediates, the viral genome itself, and/or viral transcriptions (Marques et al., 2013). These virus related dsRNAs, are processed into 21~22 nt siRNAs by Dicer, then these siRNAs are loaded onto Argonaute (Ago) forming the RISC with other proteins (Wilson and Doudna, 2013). One of the strands of siRNAs is selected and serves as a sequence-specific guide to cleave target viral mRNA (or genome) by complementary binding (Wang et al., 2015). However, in mammalian species, these viral derived long dsRNAs generally induce a protein-based innate antiviral immune response called the interferon (IFN) response to control viral infection, which supplants the siRNA pathway antiviral response (Cullen, 2014). This pathway was discovered from studies on the role of IFN in the control of immune response in vertebrates, but it is now recognized to play a very important role in the regulation of both innate immune and adaptive immune systems (O'Shea and Plenge, 2012; Stark and Darnell Jr, 2012). As a protein-based innate antiviral immune pathway, JAK/STAT has also been reported to control various viruses in

insects (Dostert et al., 2005; Paradkar et al., 2012; Souza-Neto et al., 2009). In insect, the JAK/STAT pathway is generally initiated by the Upd binding to the transmembrane receptor Dome which is a distant homolog of the vertebrate type I cytokine receptor. The conformational change of Dome after Upd binding leads to the self-phosphorylation of the Hop. The activated Hop will phosphorylate Dome, thereby forming docking sites for the cytoplasmic STATs. The recruitment of STAT to these docking sites enables Hop to phosphorylate STAT which leads to its dimerization. Subsequently, the STAT dimers translocate to the nucleus where they activate transcription of specific effector to control viruses (Myllymäki and Rämet, 2014).

From mammals to insects, the studies of antiviral responses partially disclosure the evolution of the innate immune pathways, especially those RNAi and protein based. Unlike mammals, insects without adaptive immunity may not only linearly rely on RNAi and protein based immune pathways, but also the communications among them. Therefore, the communications between nucleotides-based and protein-based antiviral strategies are currently an interest in insects. Intriguingly, in the well-studied model insect mosquito, the siRNA pathway communicates with the JAK/STAT pathway during viral infection (Kingsolver and Hardy, 2012; Paradkar et al., 2014; Paradkar et al., 2012). As shown in Figure 1, upon viral infection, the upregulation of *Dicer-2* of the siRNA pathway leads to the activation of *Vago* transcription, which increases the level of secreted Vago. Subsequently, Vago induces the JAK/STAT antiviral immunity in neighboring uninfected cells, which is similar to mammalian interferon (Paradkar et al., 2014; Paradkar et al., 2012).

Viruses, being often transmitted between domesticated and wild bees (Fürst et al., 2014; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010), are a possible driver of bee declines (Goulson et al., 2015; Potts et al., 2010). Bee viruses are mainly from the families of

Dicistroviridae and *Iflaviridae*, both in the order of *Picornavirales*, which are non-enveloped small icosahedral virions, covering a positive sense single stranded RNA genome. In both honeybees and bumblebees non-specific dsRNA can induce a noticeable antiviral activity (Flenniken and Andino, 2013; Piot et al., 2015), which may suggest the nucleotides-based antiviral response goes beyond the siRNA pathway and involves other pathway with antiviral activitys in bees. In addition, studies in honeybee showed some differentially expressed genes associated with the siRNA, Toll, JAK/STAT pathways upon IAPV infection (Chen et al., 2014; Galbraith et al., 2015). Intriguingly, bees from different spectrum of sociality ranging from solitary to social bees, they all have a rather small immune genes repertoire (Barribeau et al., 2015; Kapheim et al., 2015; Sadd et al., 2015) in comparison with the super model insect *Drosophila*, which indicate the evolution of bee immunity predates the evolution of sociality (Barribeau et al., 2015; Kapheim et al., 2015). Therefore, with the model of bumblebees, *Bombus terrestris*, which have a small immune genes repertoire and stand in the middle of eusociality, we first analyzed the involvement of JAK/STAT upon viral infections and then comprehensively investigated whether Vago could communicate the siRNA with JAK/STAT pathways *in vivo* by taking gene expression as indictor, upon three setups, including viral infection (IAPV from *Dicistroviridae* as virulent infection and SBPV from *Iflaviridae* as avirulent infection), core genes depletion (*Dicer-2*, *Vago*, and *Hop*), and combinations of viral infection and core genes depletion.

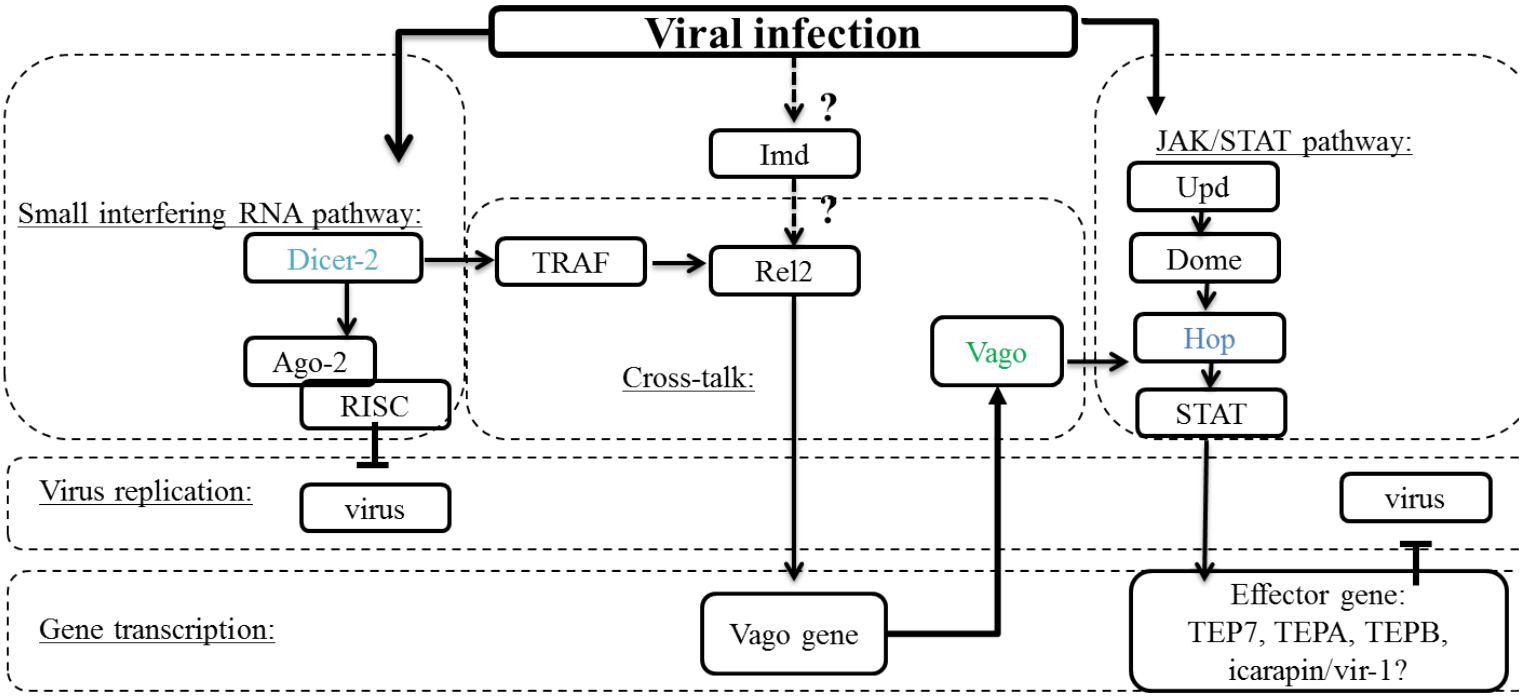


Figure 1: **Characterization of the cross-talk between the siRNA and JAK/STAT pathways in the model of mosquito upon viral infections (Paradkar et al., 2014; Paradkar et al., 2012; Souza-Neto et al., 2009).**

During viral infections, the siRNA pathway and JAK/STAT pathway are directly involved to combat viruses. In addition, a Vago related cross-talk between these two pathways upon viral infections also exists in the manner of Dicer-2 and Hop dependent. Activated STATs transcriptionally regulate antimicrobial effectors TEP7, TEPA and TEPB. Although *vir-1* is the downstream transcribed gene of JAK/STAT during virus infection (Deddouche et al., 2008; Dostert et al., 2005), the protein sequence of vir-1 in *Drosophila* and mosquito showed a significant similarity to the newly identified protein in bees, namely icarapin, a novel IgE-binging venom protein, which can evoke an immune response in subjects after a bee sting (Peiren et al., 2006).

2. Material and Methods

2.1. Insects and viruses

Newly emerged workers were collected from the colonies of *B. terrestris* provided by Biobest NV (Belgium), and kept in micro-colonies fed with pollen and sugar water *ad libitum* in incubator (Panasonic) under the condition of 29-31°C, 60-65% relative humidity, and continuous darkness, for further experiments. Colonies used in this study were screened to be free of IAPV and SBPV infections by RT-PCR (supplementary data: Table S1). IAPV and SBPV inocula were produced by following the protocol described in a previous study (Niu et al., 2014a). The exact virus particles were counted by using transmission electron microscopy. These two viruses were chosen based on their virulence in bumblebees in our setup: IAPV (virulent virus) presents an extremely fast replication and causes high mortalities of the bees within few days; SBPV (avirulent virus), its infection causing no mortality, still replicates fast but slower compared with that of IAPV (Chapter II Figure 1). The bees used in our experiments were five to eight days old, except the experiment of IAPV feeding (two days old worker were used), all the RNA samples were collected in less than 11 days old adult bees.

2.2. Viral inoculations

We used ~20 particles (in 5 µl solution) of IAPV per bee to apply the injection. The amount of SBPV is ~200, 000 particles (in 5 µl solution) per bee for injection. PBS injected bees served as control. Before the injection, bees were transferred into 50 ml tube and incubated on ice for ~20 min. Then, the unconscious bees were immediately injected with virus by the nano-injector. To maintain an accurate injection and avoid any leak of injection solutions, we chose the soft white-

like cuticle between the 1st and 2nd segments as injection site and the injection process were strictly screened under microscope. The injected bees were immediately transferred back to micro-colonies with same condition as described above. When IAPV feeding was required, bees with 5 hours starvation were transferred into a petri dish, and a liquid drop (in 20 µl solution) containing of an amount of 10^8 particles (mixed with sugar water instantly before feeding) were ingested per bee. Only bees that directly and completely ingested the solution within one hour were put back to micro-colonies. The control treatment was followed with the same procedure but with PBS spiked sugar water.

2.3. Gene silencing by dsRNA

A fragment of target gene was amplified by PCR with target gene sequence specific primers plus T7 promoters (supplementary file: Table S2). Then, these partial DNA templates of each gene were purified by E.Z.N.A.® Cycle-Pure Kit (Omega, GA). The specificity of each template was checked by running the PCR products in an electrophoresis on 1.5% agarose gel and sequence confirmations for these templates (LGC genomics, Berlin, Germany). Next, one microgram templates were used to synthesis dsRNA according to the guideline of MEGAscript® RNAi Kit (Invitrogen, USA). The concentration and quality of each dsRNA were verified by Nanodrop and electrophoresis on 1.5% agarose gel. With the same procedure, a partial of GFP sequence (Supplementary file: S2) was used as template to synthesis dsGFP as negative control. For each gene silencing, a total of 20 µg (in a volume of 20 µl) of dsRNA were injected per bee, and same dose of dsGFP was served as negative control for the effect of non-specific dsRNA.

2.4. Samples preparation for exploring the roles of *Bt*Vago in communications between the siRNA and JAK/STAT pathways

To comprehensively investigate the possible roles of *Bt*Vago in communications between the

siRNA and JAK/STAT pathways, we chose the expression of *Bt*Dicer-2, *Bt*Vago and *Bt*Hop, as the indicator to follow the interactions for the pathways, upon three experimental setups. The "Setup I" focused on the influence of the single factor "virus infection" on the expressions on *Bt*Dicer-2, *Bt*Vago, and *Bt*Hop; The "Setup II" was about silencing one of these three genes and detected its influence on the other's expressions; The "Setup III" combined the above two factors, viral infection and gene silencing. The details of sample collections were described in following:

Setup I: gene expression upon viral infections. Two days after injection of IAPV, the whole body of the bee was collected for RNA extraction (n= 10), the expression of *Bt*Dicer-2, *Bt*Vago and *Bt*Hop was compared with control bees (n = 10) undergoing the same procedure except injected with PBS. For IAPV infection by feeding, we collected bees after 9 days after IAPV feeding, and the abdomen of each individual (n=8) was used to extract RNA. PBS fed bees (n=8) were used as controls. For SBPV, after 3 days injection, the abdomen of each bee was used for RNA isolation (n=8), and the same treatment of PBS injection was used as control (n=8).

Setup II: gene expression upon gene silencing. Gene silencing was performed by injection of gene specific dsRNA to target mRNA of *Bt*Dicer-2, *Bt*Vago, and *Bt*Hop, respectively. Two days after dsRNA injection, the abdomens of each individual were collected for RNA extraction. DsDicer-2 (n=10), dsVago (n=4), and dsHop (n=10) were applied to silence *Bt*Dicer-2, *Bt*Vago, and *Bt*Hop, respectively. For each gene silencing, dsGFP were used as negative control.

Setup III: gene expression in combined effect on gene silencing and viral infections. This setup combined the factors of gene silencing (*Bt*Dicer-2 and *Bt*Vago) and viral infections (IAPV and SBPV). We first silenced the genes through injection with sequence specific dsRNA, after two days, we inoculated bees with SBPV or IAPV by injection. Subsequently, post two days of SBPV injection and post 1.5 days IAPV infection, RNA for each groups were collected. For

group of SBPV, two treatments were applied, dsDicer-2-SBPV (n=8) and dsVago-SBPV (n=15). For groups of IAPV, two groups were involved: dsDicer-2-IAPV (n=18) and dsVago-IAPV (n=9). In each treatment, dsGFP controls were applied as the controls.

2.5. Virus genome copy number detection

To measure whether the silencing of *Bt*Hop and *Bt*Vago could influence the amount of IAPV and SBPV gcn, the relative viral genome copies in each sample were evaluated based on a DNA standard curve. A part of the each virus genome was amplified (primers in supplementary table S1) and purified by E.Z.N.A.® Cycle-Pure Kit . The sequences of each virus were confirmed by Sanger sequencing. The concentration of purified templates was measured by Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, USA). The concentration was converted to gcn per µl by the online tool (URL: http://cels.uri.edu/gsc/cndna.html; Accessed date: 20 June 2014). A serial 10 times dilution of templates was made to obtain a standard curve for each virus by qPCR. With cq values (x) and corresponding gcn (y), for IAPV, the equation is $y = -0.3017x + 8.8995$ ($R^2 = 0.9997$); for SBPV, the equation is $y = -0.2926x + 9.4426$ ($R^2 = 0.9996$). The normalized gcn of each sample was represented by the ratio of the gcn calculated that is based on the DNA standard curve and the normalization factor from the internal reference gene peptidylprolyl isomerase A (*PPIA*) (Niu et al., 2014a) with the frame work of qBase (Hellemans et al., 2007).

2.6. Mortality test

To further test whether silencing of *Bt*Hop would lead to a change of mortality caused by virus infection, the mortalities of *Bt*Hop silenced bees under viral infection were followed for around six weeks. Each treatment included 15 biological replicates, and the mortality was checked per day. The dead bees were removed out of micro-colonies after scoring the mortality.

2.7. RNA isolation, cDNA, and qPCR

RNA isolations were applied by RNeasy mini kit (Qiagen, Germany). Extra RLT buffer (1.5~2 ml) were used to homogenize the lysed bumblebee tissues by mortar and the supernatants were centrifuged for three times to remove the deposit, afterwards the steps were followed by the standard protocol of kit. Then, RNA was treated by TURBO DNA-free™ kit (Ambion, USA) to remove possible genomic DNA. RNA quantity and quality were checked by Nanodrop and electrophoresis on 1.5% agarose gel. Two microgram RNA was used to synthesize the cDNA by SuperScript® II Reverse Transcriptase (Invitrogen, USA) using oligo (dT) primers. To make sure that genomic DNA was removed completely we checked cDNA samples with exon spanning primers for RPL23 (Supplementary data Table S1). The cDNA should produce an amplicon of 143 bp while possible genomic DNA contamination would produce an extra amplicon of 452 bp. The qPCR was performed on a CFX96™ Real-Time PCR Detection using GoTaq® qPCR master (Promega, USA). Each reaction was performed in duplicate. The amplification specificity of primers used in this study was checked by both electrophoresis of the RT-PCR products and analysis of the dissociation curve of qPCR. Each RT-PCR products amplified by these primers were sequenced in order to confirm their primers' specificities.

3. Results

3.1. JAK/STAT pathway in *B. terrestris* is involved in the control of SBPV

To identify the involvement of the JAK/STAT pathway upon virus infection, we looked at whether silencing of *Bt*Hop (one of key components of JAK/STAT pathway) would change the infection types of viruses, such as causing higher mortality. The results showed that the silencing of *Bt*Hop did not increase the mortalities upon both viral infections (Figure 2). Then, we wondered whether there was any difference in the viral gcn by the silencing of *Bt*Hop. The results revealed that silencing of *Bt*Hop did not influence the amount of

IAPV gcn compared with dsGFP control (Figure 3A). However, it significantly increased the amount of gcn of SBPV at two dpi (T-test: t=-2.683, df=19, *p=0.015*), but at three pdi, there was no significant difference of gcn compared with dsGFP control (Figure 3B). Thus, these results suggest the involvement of the JAK/STAT pathway against SBPV infection, but the temporal silencing of *Bt*Hop did not alter the SBPV infection type, such as from a covert to an overt infection.

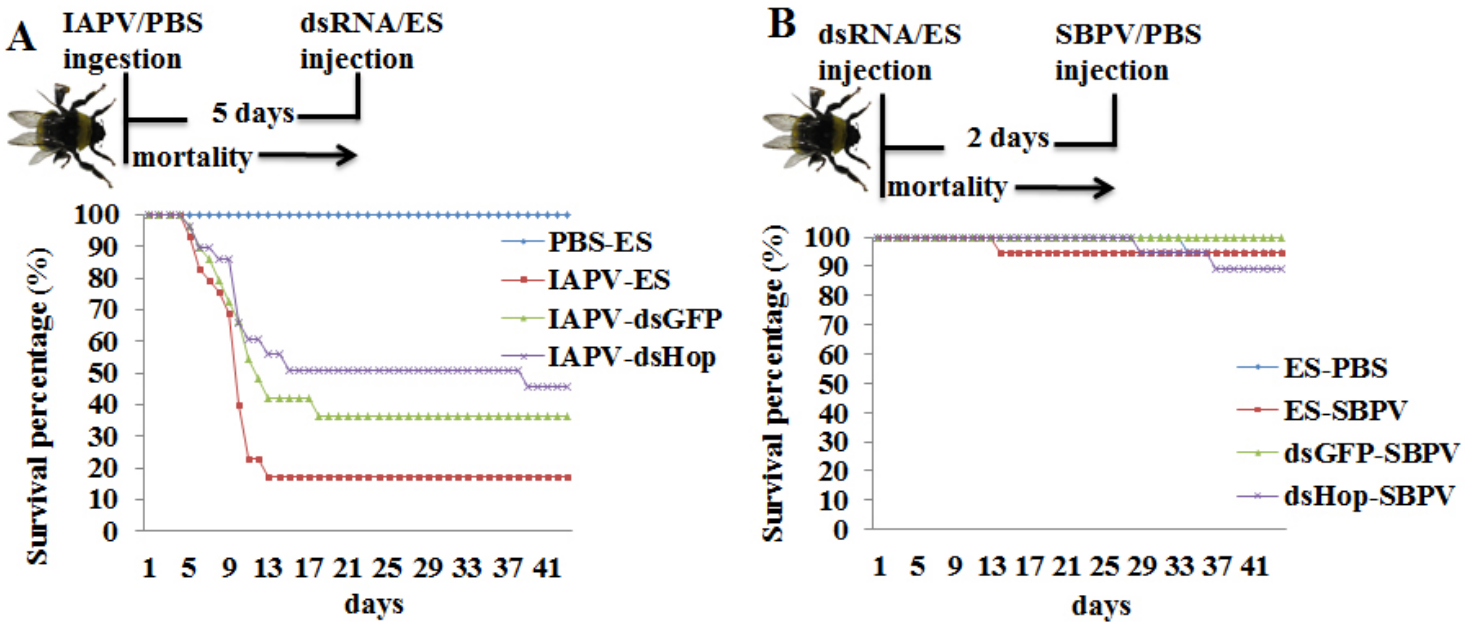


Figure 2: Survival percentage of bumblebees caused by virus infection upon silencing of core JAK/STAT pathway gene: *Bt*Hop.

(A) IAPV: The injection of IAPV causes extremely high and fast mortality, therefore, we used feeding as the inoculation method. IAPV was firstly ingested by fixed age adult bees, and then after five days later, dsRNA were injected to silence *Bt*Hop. DsGFP and ES were included as the controls. (B) SBPV: DsRNA were firstly injected to silence *Bt*Hop in age fixed adult bees. After two days later, we injected SBPV to infect bumblebees. For all treatments including controls, at least 15 biological replicates were used and the mortalities of bees were checked per day.

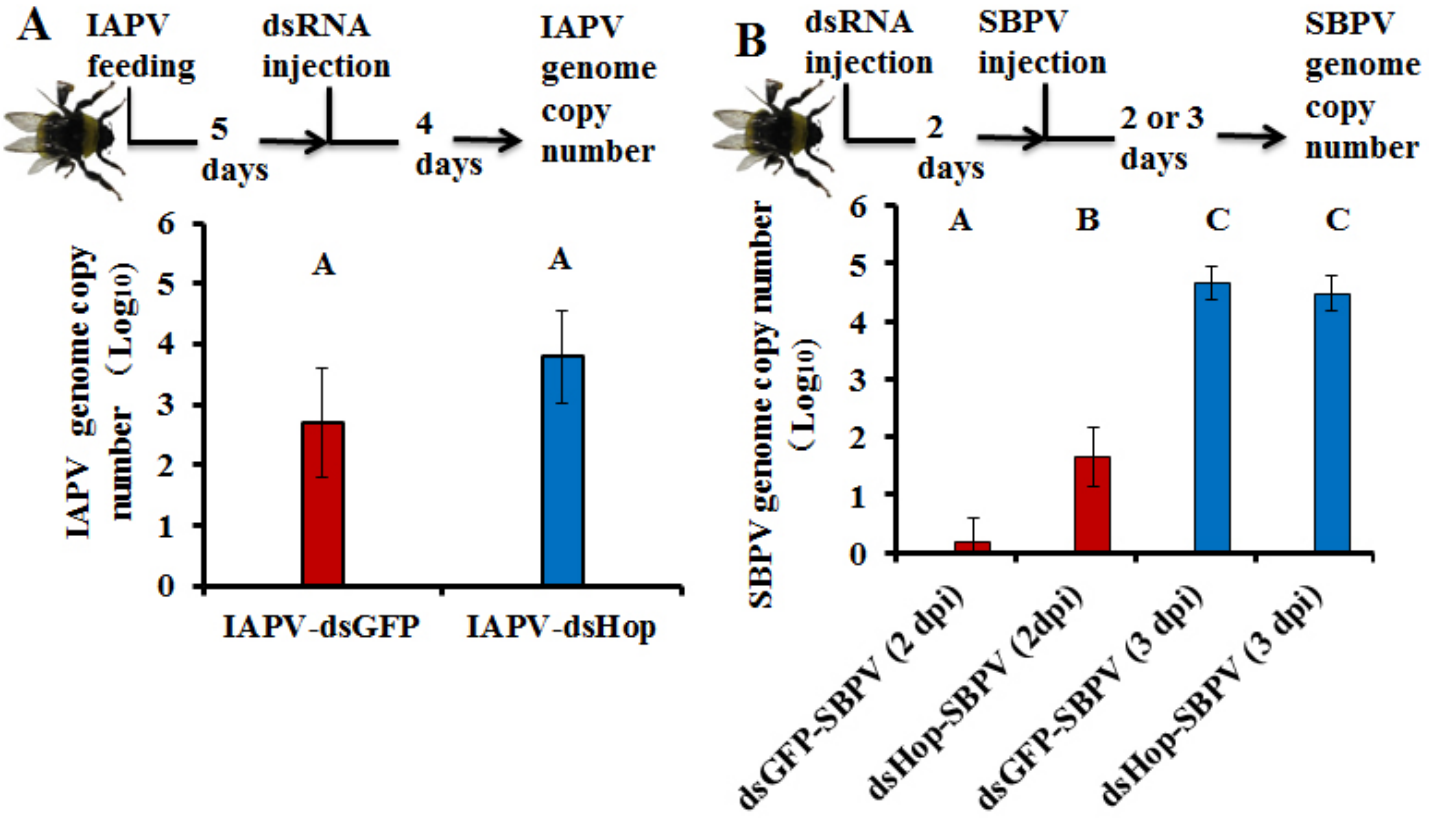


Figure 3: Genome copy numbers of viruses upon silencing of core JAK/STAT pathway gene: *Bt*Hop.

(A) IAPV: The injection of IAPV causes extremely high and fast mortality, therefore, we used feeding as the inoculation method. IAPV was firstly ingested by fixed age adult bees, and then after five days, dsRNA were injected to silence *Bt*Hop. DsGFP and ES were included as the controls. Subsequently, post four days injection of dsRNA, genome copy number of IAPV was measured. (B) SBPV: dsRNA were firstly injected to silence *Bt*Hop in age fixed adult bees. After two days later, we injected SBPV to infect bumblebees. Subsequently, post two and three days injection of dsRNA, genome copy number of SBPV was measured. At least 8 biological replicates were included in each treatment. The means of each genome copy number were represented based on Log_{10} transformation. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p< 0.05$ was labelled with asterisks (*).

3.2. The orthologues of Vago in *B. terrestris*

An orthologue of mosquito Vago was identified in the genome of *B. terrestris*, namely, *Bt*Vago (XP_003399812.1). The 153-aa protein sequence of *Bt*Vago showed 69% identity with *Cx*Vago (Vago of *Culex quinquefasciatus*: XP_001844380.1) based on NCBI-Blast. *Bt*Vago presented eight conserved cysteine residues which form a von Willebrand factor C-domain (VWC) (Figure 4). SingalP 4.1 (Petersen et al., 2011) analysis of the *Bt*Vago amino acid sequence indicated a predicted signal peptide cleavage site between amino acids 16 and 17 (Figure 5). This suggests that *Bt*Vago may also be secreted and has an IFN-like antiviral function as described in mosquito (Paradkar et al., 2014; Paradkar et al., 2012). Phylogenetic analysis of Vago-related protein sequences revealed that insects Vago mainly followed their taxonomy (Figure 6). Based on the VWC domain as a template from Protein Data Bank (PDB code 1U5M), the proposed 3D protein structure of *Bt*Vago and *Cx*Vago were constructed by Swiss-model (http://swissmodel.expasy.org/interactive accessed on: April 22, 2015), while only seven conserved cysteine residues of each Vago were aligned with the 1U5M during the model constructions (Figure 6). In order to identify the possible promoter region responsible for *Bt*Vago activation, the 5' regions ~2 kb upstream from the transcription start site of *B. terrestris* gene, was analyzed by PROMO.

Figure 4: The eight conserved cysteine residues of Vago in different insect species.

The amino acid in yellow background indicated the eight conserved cysteine residues. The conservation of amino acid at each location were indicated by *, : , ., with the level of similarity from high to low.

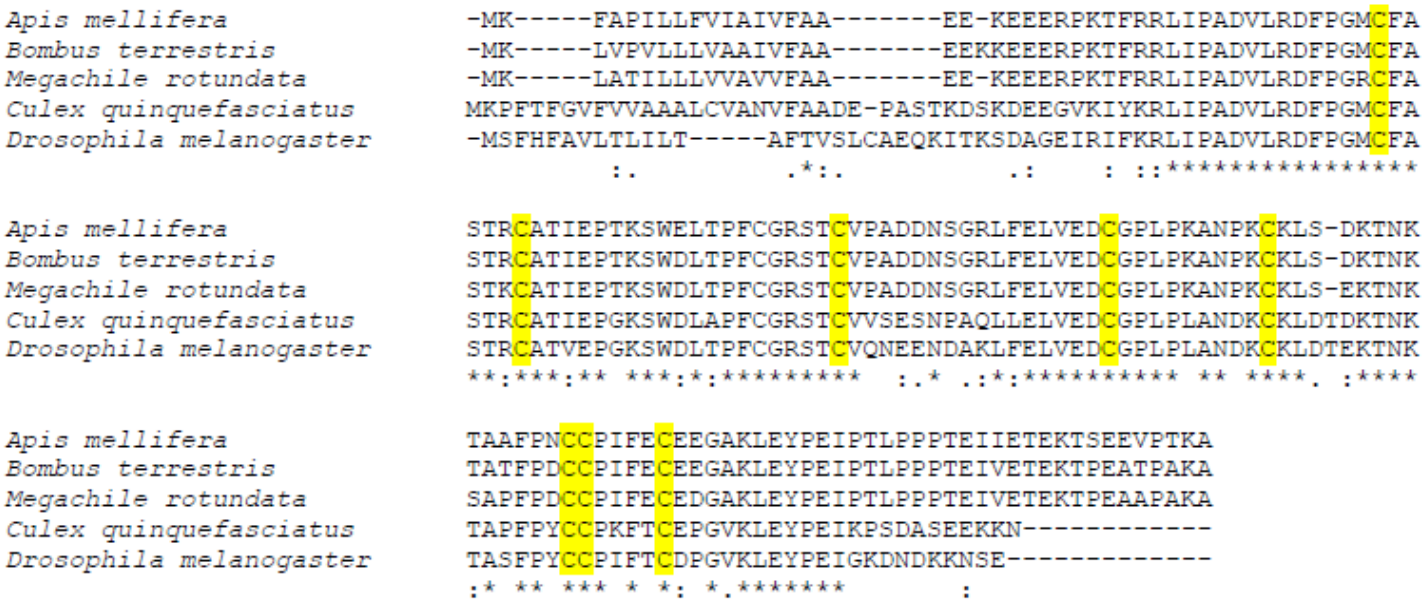
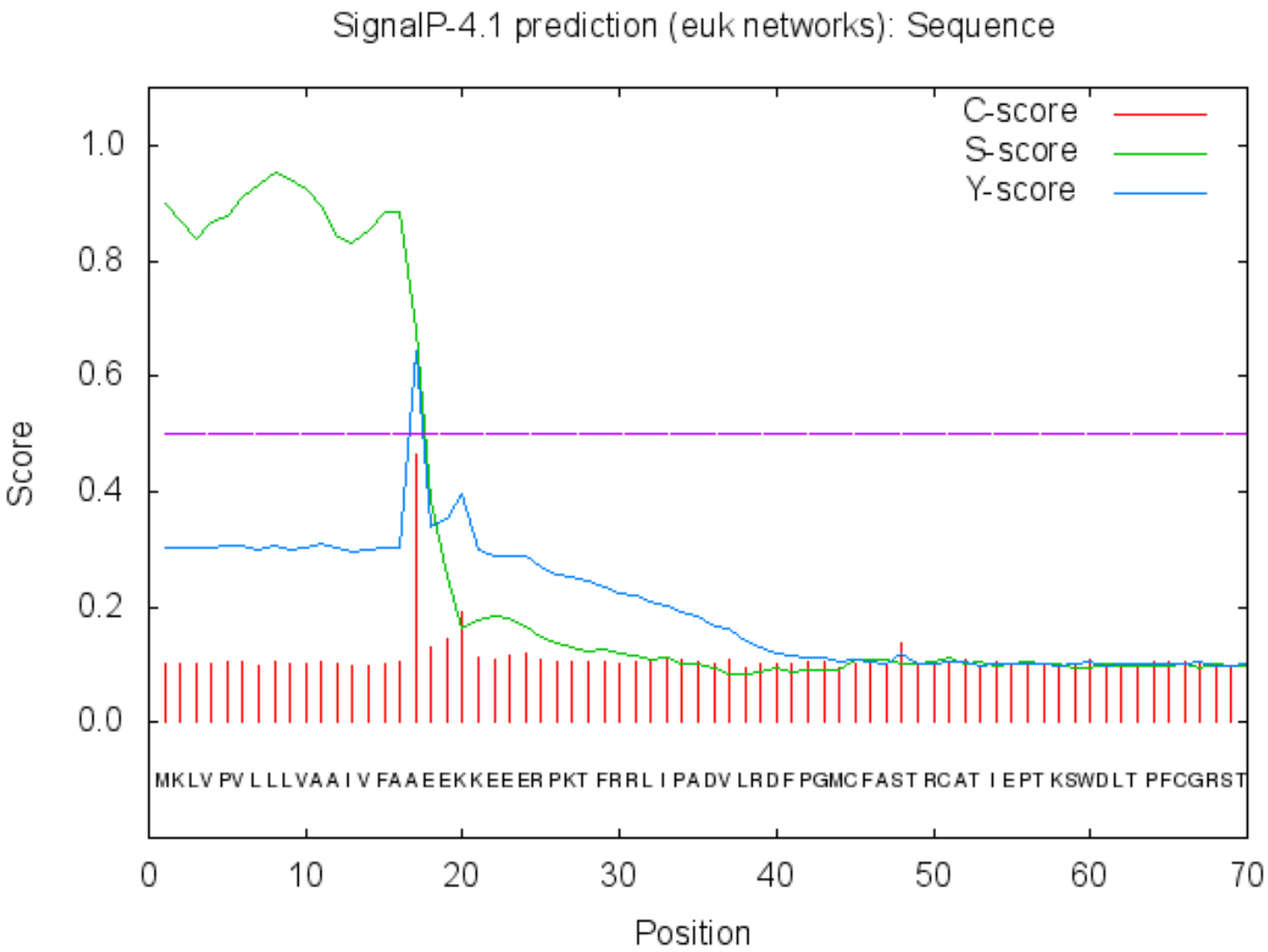


Figure 5: Predicted signal peptide cleavage site of *Bt*Vago



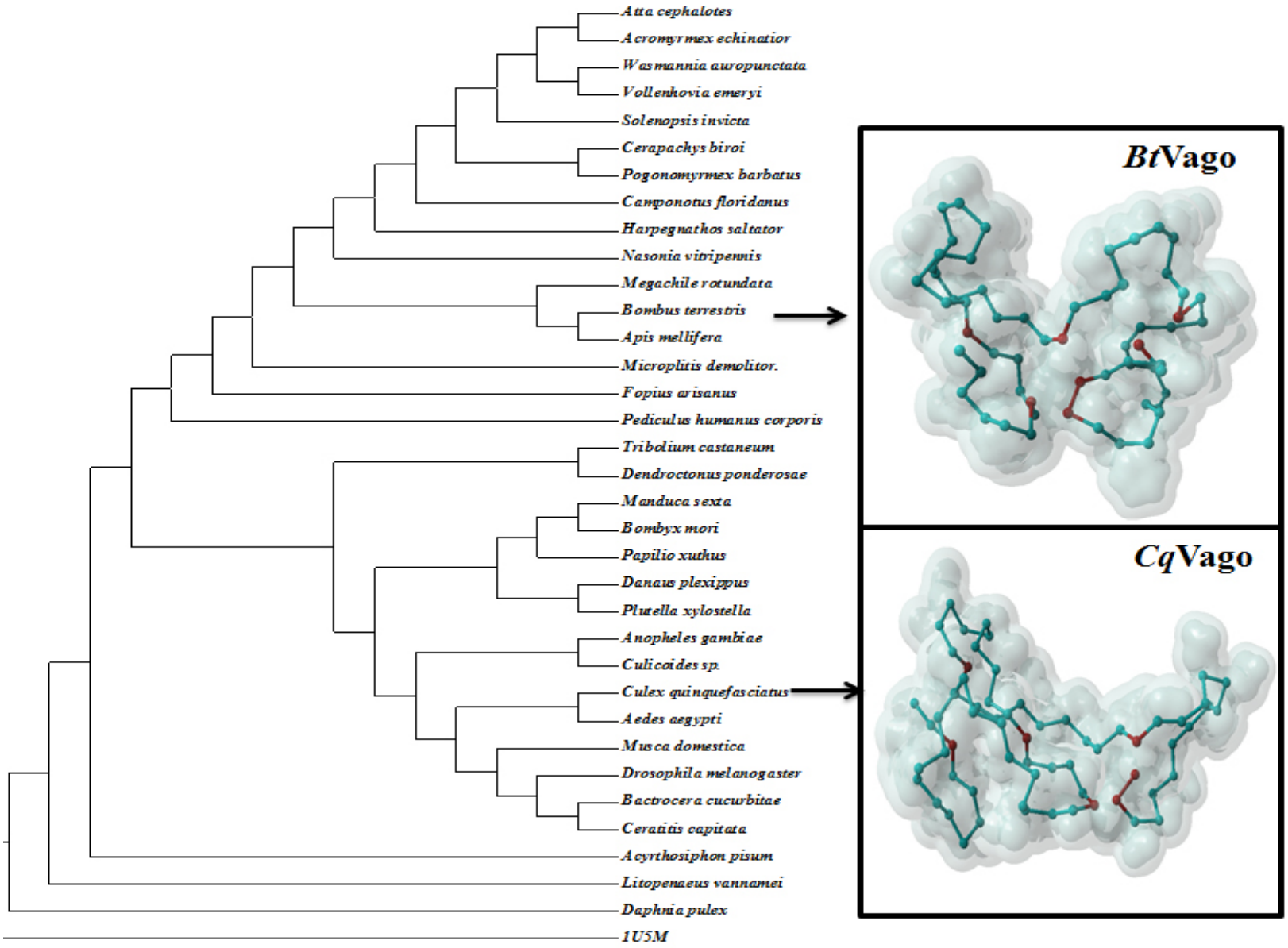


Figure 6: Phylogenetic analysis of Vago-like protein sequences and proposed 3D structures of *Bt*Vago and *Cq*Vago based on 1U5M. MEGA 6.0 (Tamura et al., 2013), was used to construct the phylogenetic tree of Vago-like proteins through Maximun Likelihood. The conserved amino acids from chosen sequences of each species were used (supplementary file: S1). The number of Bootstrap replications was 500 to test the phylogeny. The model of LG+G was adapted according to the model test of all input sequences. The proposed protein 3D structures of *Culex quinquefasciatus* Vago and *Bombus terrestris* Vago-like proteins, was constructed based by SWIS-MODEL through the template from Protein Data Bank (PDB code 1U5M). The amino acids in red shows conserved cysteine residues of each Vago were alignment with the 1U5M during the model constructions.

Table 1: Predicted NF-κB binding site in *Bt*Vago promoter region

Factor name	Start position	End position	Dissimilarity	String	RE equally	RE query
RelA [T00595]	438	448	12.115729	TCTAACGAAGC	0.02146	0.01273
GATA-1 [T00305]	735	742	4.138263	CACGATAA	0.54932	0.69044
GATA-1 [T00305]	925	932	1.520654	TTATCATT	0.30518	0.65047
GATA-1 [T00305]	1609	1616	4.052395	TCGGATAA	0.54932	0.69044
GATA-1 [T00305]	1896	1903	1.682543	TTATCACA	0.30518	0.65047
GATA-1 [T00267]	735	743	8.557309	CACGATAAT	0.06866	0.14082
GATA-1 [T00267]	924	932	4.979757	TTTATCATT	0.18311	0.36798
GATA-1 [T00267]	1609	1617	3.166737	TCGGATAAG	0.11444	0.16757
GATA-1 [T00267]	1895	1903	3.40581	CTTATCACA	0.10681	0.17821

The results indicated the presence of NF-κB biding site in *Bt*Vago promoter region (Table 1).

The above *in silico* analysis provides evidences for sequence conservation of Vago in bumblebees. In mosquito it has been reported that the siRNA pathway can induce the expression of Vago, and further lead to activate the JAK/STAT pathway, upon virus infection (Figure 1). In order to analyze this possible activity of *Bt*Vago, we comprehensively investigated the expression of associated key genes (Figure 1) including *Bt*Dicer-2, *Bt*Vago and *Bt*Hop, in three experimental setups, including upon virus infection (Setup I), upon dsRNA injection to silence one of the three genes (Setup II), and upon effects in combined viral infections with silencing of *Bt*Dicer-2 or *Bt*Vago (Setup III).

3.3. *Bt*Vago is downregulated by IAPV infection (Setup I)

In contrast to the results from mosquito showing an upregulation of *Vago* upon virus infections (Paradkar et al., 2014; Paradkar et al., 2012), our results demonstrated that the expression of *Bt*Vago was downregulated, after infection with IAPV in both inoculation ways, injection (T-test: t=-3.773, df=8.5, *p=0.005*) and ingestion (T-test: t=2.211, df=14, *p=0.044*), while at the same time *Bt*Dicer-2 was significantly upregulated (Figure 7A and B). The expression of *Bt*Vago was not changed in SBPV infected samples, although the virus could also significantly induce the expression of *Bt*Dicer-2 (Figure 7C).

3.4. Silencing of *Bt*Vago downregulates the expression of *Bt*Hop (Setup II)

Two days after the injection of gene specific dsRNA to target *Bt*Dicer-2, *Bt*Vago, or *Bt*Hop, the target genes were silenced in the comparison with controls (i.e. injection of dsGFP). Under the silencing of *Bt*Dicer-2, the expression of *Bt*Vago and *Bt*Hop was not changed (Figure 8A). The marginal silencing of *Bt*Vago significantly downregulated the expression of *Bt*Hop (T-test: t=4.889, df=6, *p=0.003*) (Figure 8B), which suggests the possible association of *Bt*Vago with the

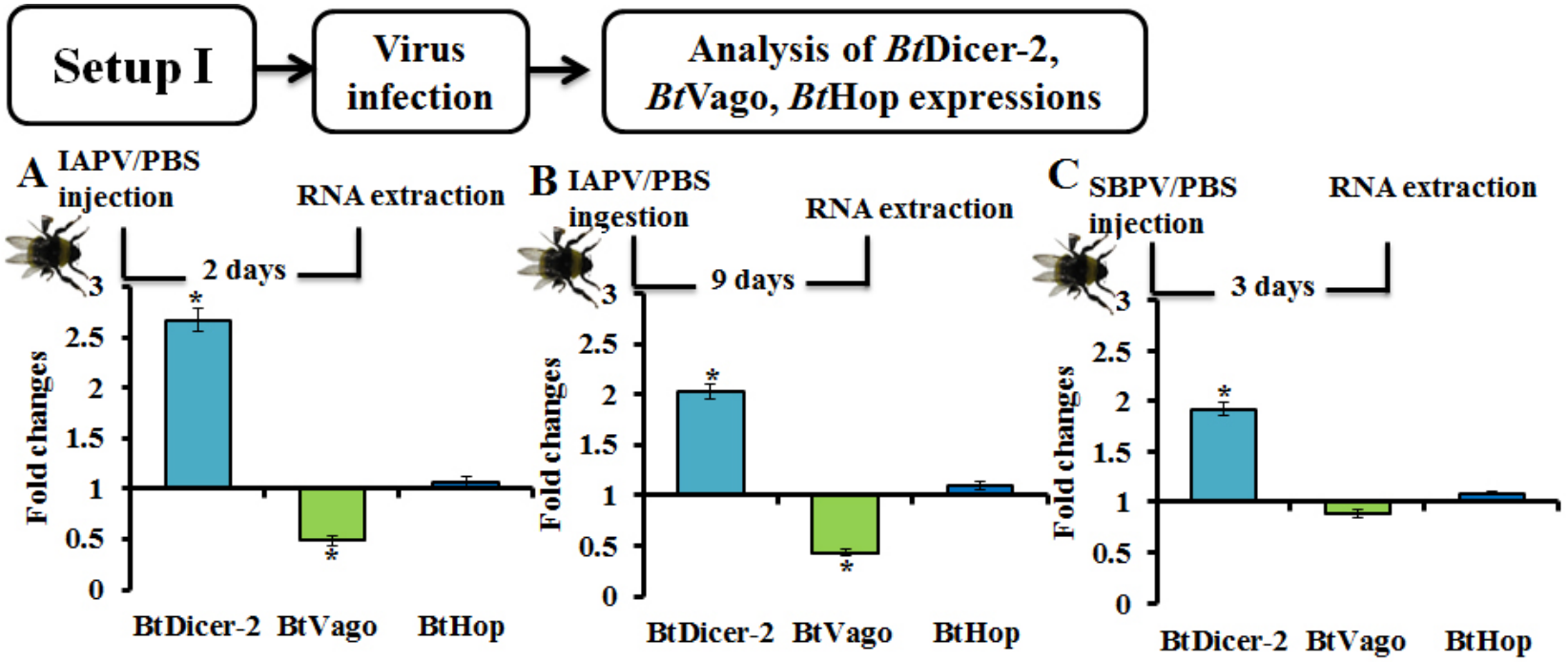


Figure 7: **Fold changes of *Bt*Dicer-2, *Bt*Vago, and *Bt*Hop upon viral infections in comparison with controls (PBS).**

(A): IAPV infection was inoculated by injection. Two days after IAPV injection, the RNA samples were collected for gene expression analysis, PBS injected samples were collected as control. (B): IAPV infection was inoculated by ingestion (feeding). Five days after IAPV ingestion, the RNA samples were collected for gene expression analysis, PBS ingested samples were collected as control. (C): SBPV infection was inoculated by injection. Three days after SBPV injection, the RNA samples were collected for gene expression analysis, PBS injected samples were collected as control. Each treatment included eight biological replicates. The fold changes of gene expression were equal to the ratio of the relative expression of each gene in virus infected samples over the relative expression of this gene in control samples (PBS injected bees). The relative expression of each gene was calculated based on internal reference gene *PPIA*. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p < 0.05$ was labelled with asterisks (*).

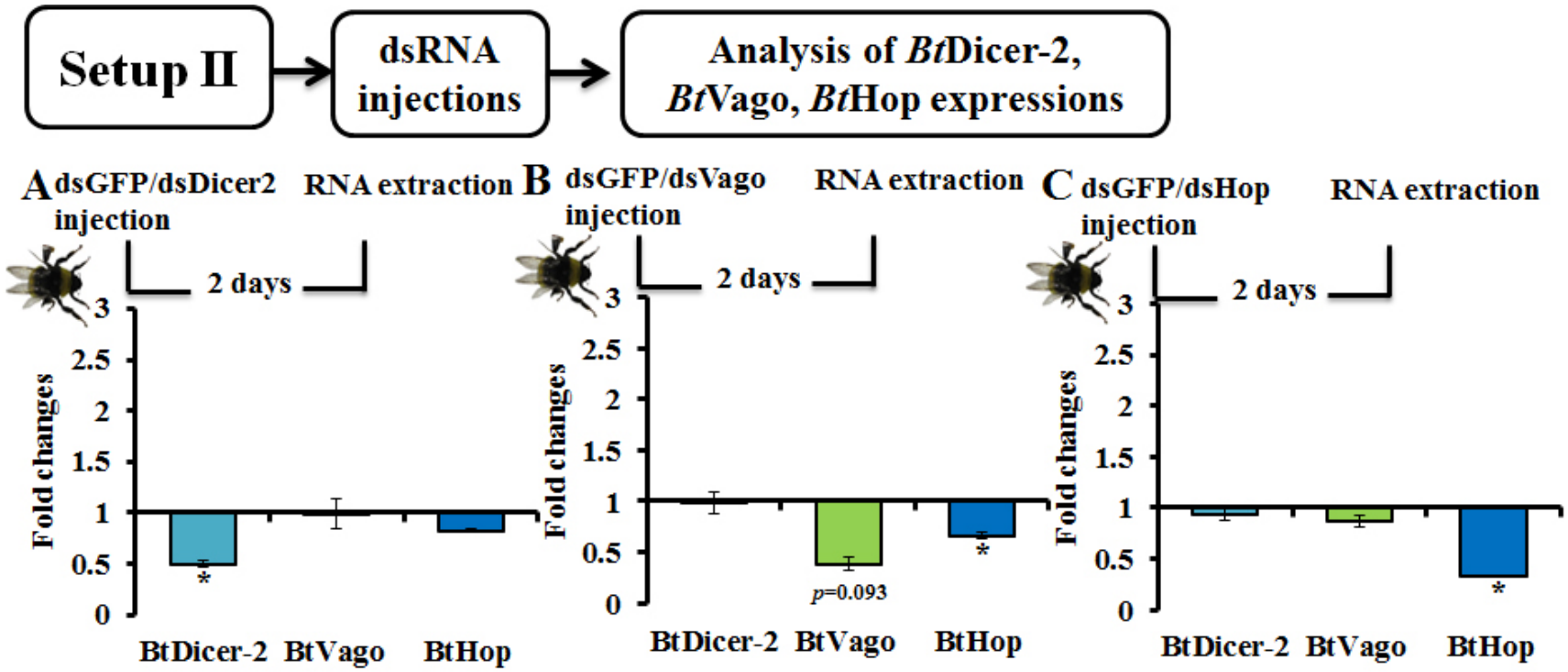


Figure 8: Fold changes of *Bt*Dicer-2, *Bt*Vago, and *Bt*Hop upon relative dsRNA injection in comparison with controls (dsGFP injection).

(A): Injection of dsDicer2 to silence *Bt*Dicer-2 (n=9~12). (B): Injection of dsVago to silence *Bt*Vago (n=4~5). (C): Injection of dsHop to silence *Bt*Hop (n=10). In each treatment, dsGFP injected samples were served as controls. All RNA samples were collected post two days injection of dsRNA. The fold changes of gene expression were equal to the ratio of the relative expression of each gene in virus infected samples over the relative expression of this gene in control samples (dsGFP injected bees). The relative expression of each gene was calculated based on internal reference gene *PPIA*. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p < 0.05$ was labelled with asterisks (*). After two days of dsRNA injection, all the target genes were downregulated compared with dsGFP controls.

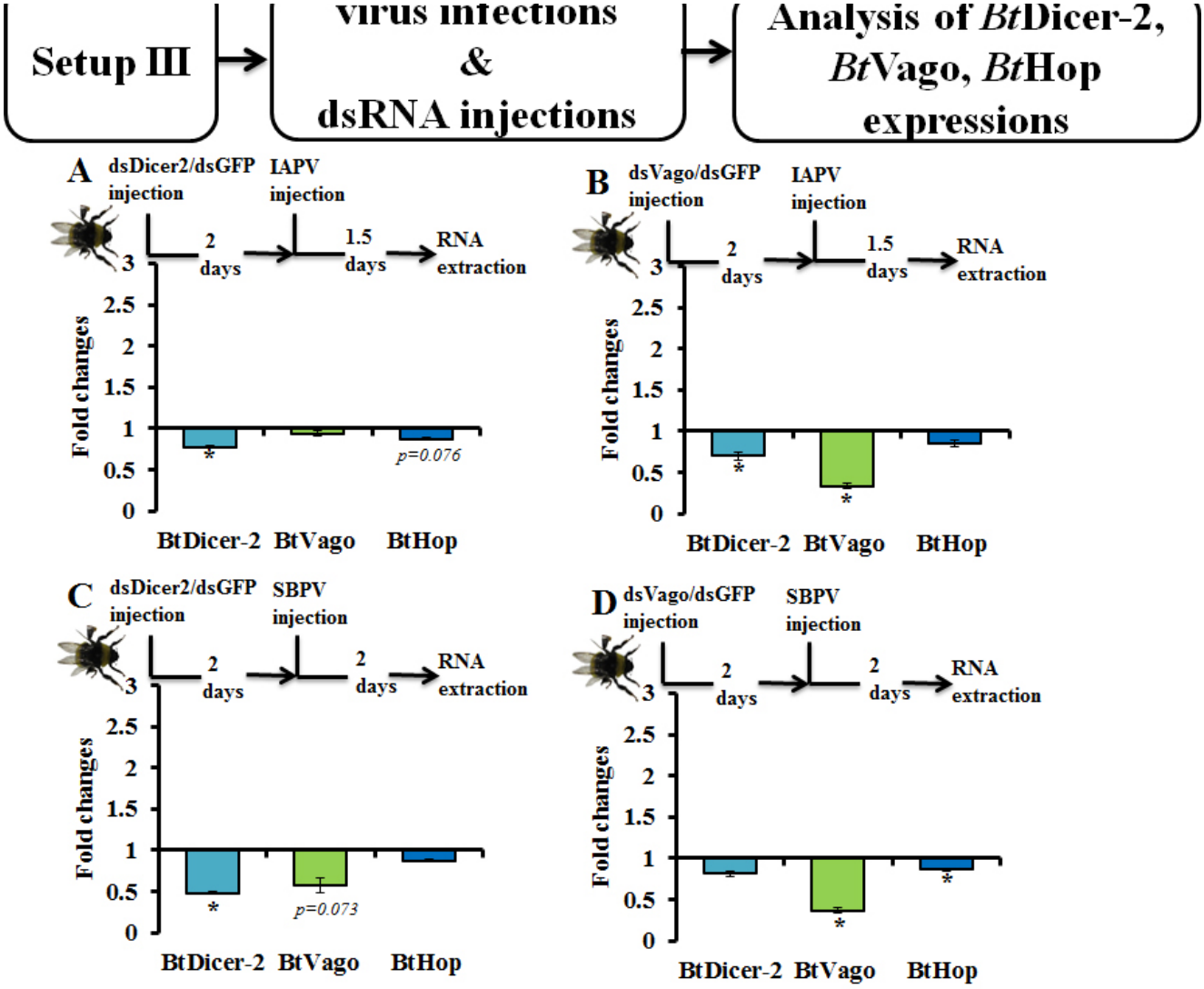


Figure 9: Fold changes of *Bt*Dicer-2, *Bt*Vago, and *Bt*Hop upon viral infections with pre-silencing of *Bt*Dicer-2 or *Bt*Vago in comparison with controls.

(A): IAPV infection with *Bt*Dicer-2 pre-silencing. DsDicer2 was injected to silence *Bt*Dicer-2, two days later, IAPV was injected to inoculate bees. Subsequently, RNA was collected post 1.5 days injection of IAPV for measuring gene expressions (n=18). (B): IAPV infection with *Bt*Vago pre-silencing. DsVago was injected to silence *Bt*Vago, two days later, IAPV was injected to inoculate bees. Subsequently, RNA was collected post 1.5 days injection of IAPV for measuring gene expressions (n=9). (C): SBPV infection with *Bt*Dicer-2 pre-silencing. DsDicer2 was injected to silence *Bt*Dicer-2, two days later, SBPV was injected to inoculate bees. Subsequently, RNA was collected post two days injection of SBPV for measuring gene expressions (n=8). (D): SBPV infection with *Bt*Vago pre-silencing. DsVago was injected to silence *Bt*Vago, two days later, SBPV was injected to inoculate bees. Subsequently, RNA was collected post two days injection of SBPV for measuring gene expressions (n=15). We used dsGFP and PBS injections as controls for dsRNA and viral injections, respectively, for each treatment. The fold changes of gene expression were equal to the ratio of the relative expression of each gene in virus infected samples over the relative expression of this gene in control samples. The relative expression of each gene was calculated based on internal reference gene *PPIA*. The error bar represented the standard error of mean. The level of significance was calculated by T-test and $p< 0.05$ was labelled with asterisks (*).

JAK/STAT pathway. We did not detect any influence on the expression of *Bt*Dicer-2 and *Bt*Vago by the silencing of *Bt*Hop (Figure 8C).

3.5. Combined effects (the *Bt*Dicer-2 or *Bt*Vago depletion, and the viral infections) show the proposed communications between the siRNA pathway and JAK/STAT pathway (Setup III)

With the evidences of viral infection in influence the expressions of *Bt*Dicer-2 and *Bt*Vago (Setup I), and silencing of *Bt*Vago on influence the expression of *Bt*Hop (Setup II), we wondered whether the combination the silencing of these core genes to interfere the pathway activities, and viral infections, would show more clear evidences on the proposed communications. The results (Setup III) showed that silencing of *Bt*Dicer-2 upon IAPV infection did not influence the expression of *Bt*Vago but marginally downregulated the expression of *Bt*Hop (T-test: t=-1.856, df=24.2, *p=0.076*) (Figure 9A), and the silencing of *Bt*Dicer-2 upon SBPV infection marginally decreased the expression of *Bt*Vago (T-test: t=1.889, df=21, *p=0.073*) but no effect on the expression of *Bt*Hop was observed (Figure 9C). The silencing of *Bt*Vago upon IAPV infection significantly decreased the expression of *Bt*Dicer-2 (T-test: t=4.009, df=17.3, *p=0.001*) (Figure 9B), while the silencing of *Bt*Vago upon SBPV infection showed a significant downregulation of *Bt*Hop (T-test: t=2.153, df=18, *p=0.045*) (Figure 9D).

3.6. Silencing of *Bt*Vago did not alter the viral genome copy number

To further explore the role of *Bt*Vago to viral infections, we measured the amount of viral gcn after the silencing of *Bt*Vago. The results showed that the amount of IAPV showed no difference between treatments in dsGFP (n=15) and dsVago (n=9) (Figure 10A). The amount of SBPV gcn also remained constant between treatments of dsGFP (n=20) and dsVago (n=16) (Figure 10B).

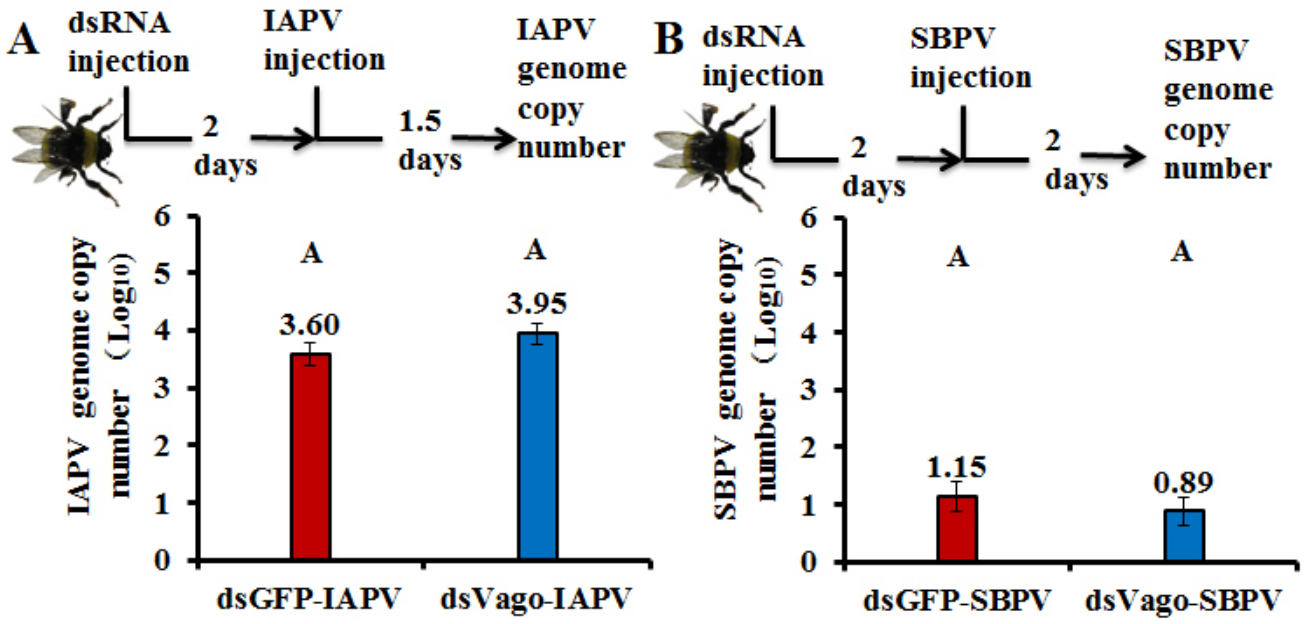


Figure 10: Genome copy numbers (gcn) of viruses upon silencing of *Bt*Vago.
(A) IAPV: DsVago (n=9) was injected to silence *Bt*Vago, two days after injection, IAPV was injected to inoculate bees. Subsequently, RNA was collected post 1.5 days injection of IAPV for measuring viral gcn. DsGFP injection was included as control (n=15). (B) SBPV: DsVago (n=16) was injected to silence *Bt*Vago, two days after injection, SBPV was injected to inoculate bees. Subsequently, RNA was collected post two days injection of SBPV for measuring viral gcn. DsGFP injection was included as control (n=20). The means of each gcn were represented based on Log_{10} transformation and the error bar represented the standard error of mean. Different letters represent statistical significant difference of mean ($p < 0.05$).

4. Discussion

In insects, especially, *Drosophila* and mosquito, the antiviral activities of JAK/STAT are well described, such as DCV, DENV and West Nile virus (WNV) (Dostert et al., 2005; Paradkar et al., 2012; Souza-Neto et al., 2009). In *B. terrestris* the JAK/STAT may also be involved in antiviral defense. Indeed when we silenced *Bt*Hop we observed an increased gcn of SBPV at 2 dpi. However the involvement of this pathway in IAPV infection is less clear, as the same approach failed to detect any influence on IAPV gcn. In wild bumblebees, IAPV and SBPV have also been

detected (Fürst et al., 2014; Levitt et al., 2013; McMahon et al., 2015; Singh et al., 2010) while viral infection symptoms in wild bumblebees have not yet been reported. Upon the artificial injection, IAPV replicates very fast and acts as a very virulent virus in bumblebees, while SBPV replicates relatively slower (data not shown) and induces no mortality. Ingestion of IAPV by the bumblebee is less harmful than injection which causes 100% mortality rapidly, but the high mortality is still observed after the ingestion of a high viral dose (Figure 2A). Since its virulence in current setup, a temporal inhibition of JAK/STAT through the silencing *Bt*Hop, may not provide a significant effect on IAPV gcn to be detected if the JAK/STAT pathway is involved in combating IAPV infection in bumblebee.

The induction of core genes in the siRNA pathway upon virus infection is detected in the honeybee (Galbraith et al., 2015) and the bumblebee, especially *Dicer-2* (Setup I). Dicer-2 belongs to the same DExD/H-box helicase family as the RIG-I-like receptors, which sense viral infection and mediate interferon induction in mammals (Deddouche et al., 2008). In studies from *Drosophila* and mosquito, it was shown that Vago can be induced in a Dicer-2 dependent manner upon viral infection (Deddouche et al., 2008; Paradkar et al., 2014; Paradkar et al., 2012). However, upon both injection and ingestion of IAPV, the expression of *Bt*Dicer-2 was increased but meanwhile the expression of *Bt*Vago was deceased. To the best of our knowledge this is the first time to report the downregulation of *Vago* in insects upon virus infection. The downregulation of *Bt*Vago is rather linked with IAPV than with *Dicer-2* upregulation since the other virus, SBPV, resulted in upregulation of *Bt*Dicer-2 while the expression of *Bt*Vago is not altered. In *Drosophila*, Vago is induced after viral infection like DCV and SINV, but no effect has been reported from flock house virus (FHV) (Deddouche et al., 2008). These evidences suggest that the induction of Vago, may be dependent on the specific virus and virus–host

interaction.

Vago appears to be a cytokine that acts in a similar manner to mammalian interferon to induce the antiviral activity of the JAK/STAT pathway in neighboring cells (Paradkar et al., 2014; Paradkar et al., 2012). The silencing of *Cq*Hop, but not *Cq*Dome, influences the *Cq*Vago induced suppression of WNV replication (Paradkar et al., 2012). Therefore, the silencing of *Bt*Vago led to a downregulation of *Bt*Hop in *B. terrestris* may imply that there could be an interaction of *Bt*Vago with the JAK/STAT pathway in *B. terrestris* and the relative small drop of *Bt*Hop expression could be estimated as the partial activity of the JAK/STAT pathway associated with *Bt*Vago. Infection of different bacteria can also induce the expression of Vago but does not influence the expression of *vir-1* being the downstream transcribed gene of JAK/STAT during virus infection (Deddouche et al., 2008; Dostert et al., 2005). Intriguingly, the protein sequence of vir-1 in *Drosophila* and mosquito only showed a significant similarity to the newly identified protein in bees, namely icarapin, a novel IgE-binging venom protein, which can evoke an immune response in subjects after a bee sting (Peiren et al., 2006). Therefore, the transcribed genes in the JAK/STAT pathway of bees upon virus infection await further investigations.

The silencing of *Bt*Dicer-2 led to a lower expression of *Bt*Hop with a noticeable level ($p=0.076$) in IAPV infected bees, while it led to the decrease of *Bt*Vago with a noticeable level ($p=0.073$) under the infection of SBPV. Although we lack the evidence of a relatively altered expression of *Bt*Vago and *Bt*Hop in IAPV- and SBPV-infected bumblebee, respectively, it seems that the silencing of *Bt*Dicer-2 could partially influence the "*Bt*Vago-JAK/STAT (*Bt*Hop)" association upon virus infection. If we take into consideration that the gene expression was performed *in vivo* and on whole abdomens, we could argue that some apparent effect can be missed. Nevertheless, these evidences may still be consistent with the proposed function of Vago as

across-talk molecule between the siRNA pathway and the JAK/STAT pathway in a Dicer-2 dependent manner in *Drosophila* and mosquito.

Upon IAPV infection the silencing of *Bt*Vago can influence the expression of *Bt*Dicer-2 (Fig 9A). Together with the fact that IAPV induces lower expression of *Bt*Vago, could this lower expression of Vago be a virus strategy not only to silence JAK/STAT defense, but also the siRNA antiviral immunity? To verify the biological context of the downregulation of Vago to viral infection, we silenced *Bt*Vago to detect its impact on IAPV and SBPV infection. In contrast to our expectations it did not show any influence on virus gcn. However, in other studies, the silencing of Vago or the mutation of Vago could lead to an increase of virus titers (Deddouche et al., 2008; Paradkar et al., 2012).

Taken all evidences together (as summary in Table 2), the interaction between the siRNA and JAK/STAT pathways through *Bt*Vago could be more complex in bumblebee compared to recent reports in *Drosophila* and mosquito. Indeed we observed that the innate immunity is not static and the role of Vago is highly dependent on the viral infections. Here we formulate some speculations on how the different types of viral infections influence *Bt*Vago in associations with communications. One, *Bt*Vago severs as the cross-talk bridge between the siRNA pathway and the JAK/STAT pathway in a similar way as described in mosquito (Paradkar et al., 2014; Paradkar et al., 2012). However this classical cross-talk could be complex upon infection of virulent virus such as IAPV. We can ask whether this downregulation of *Bt*Vago by IAPV is of interest to the host or the virus. At first glance one would say beneficiary to the virus via partially shutting off *Bt*Vago associated communications of the siRNA and JAK/STAT pathways. However, it could also be beneficiary to the host because switching off the induction of the extra activities of JAK/STAT may be able to open opportunities for other pathway with antiviral

activitys.

Table 2: Summary of differential expressions of *Bt*Dicer-2, *Bt*Vago and *Bt*Hop under three setups

Experimental design	Treatment		Gene expression *Bt*Dicer-2	*Bt*Vago	*Bt*Hop
Setup I: Viral infection	IAPV (injection)		up	down	-
	IAPV (ingestion)		up	down	-
	SBPV (injection)		up	-	-
Setup II: Gene silencing	*Bt*Dicer-2 silencing		down	-	-
	*Bt*Vago silencing		-	down (*p=0.093*)	down
	*Bt*Hop silencing		-	-	down
Setup III: viral infection & Gene silencing	*Bt*Dicer-2 silencing	IAPV(injection)	down	-	down (*p=0.076*)
	*Bt*Dicer-2 silencing	SBPV(injection)	down	down (*p=0.073*)	-
	*Bt*Vago silencing	IAPV(injection)	down	down	-
	*Bt*Vago silencing	SBPV(injection)	-	down	down

- Indicated that the expression of gene was no altered.

For instance, the activation of *Cq*Vago requires Relish2 (Paradkar et al., 2014) which is one of the components in the Imd pathway with antiviral activity (Costa et al., 2009). The down regulation of Vago could possibly be related with the pre-occupation of Relish2 by Imd in order to maintain high antiviral activities from Imd; Another possible speculation is that the feedback mechanism between Dicer-2 linked the siRNA pathway and Vago linked JAK/STAT pathway in *B. terrestris* upon viral infection, could be bidirectional. This is more in a "coordination" manner of both pathways to combat with viral infection. Nevertheless, since most of evidences provided in mosquito about the interaction between Dicer-2 and Vago are based on cell lines (Paradkar et al., 2012), thus the study on *in vivo* could be more complex as the antiviral activities organized in different tissues might be various, for instance, Vago might be differently regulated in different tissues, some tissues are more important in immunities. Indeed, the study of DCV infected flies revealed that *Vago* is induced in fat bodies but not in tracheae or oenocytes (Deddouche et al., 2008).

In conclusion, the innate immune pathways are important to control pathogen invasions in insects, and the communication among various immune pathways, especially nucleotides-based and protein-based, may be crucial to give rise to a complex and systemic response to pathogen challenge. In this study, we firstly showed the bumblebee JAK/STAT pathway might be involved as pathway with antiviral activity. Then we comprehensively investigated the possible communication with the siRNA and JAK/STAT pathways, through Vago, in *in vivo* bumblebee, as a primary social insect model with a small immune genes repertoire. According to the best characterized cross-talk model of the siRNA pathway and the JAK/STAT pathway through Vago in insects, we observed a similar possible association upon virus infection in bumblebees. However, some unexpected results were also observed, specifically, the downregulation of Vago

instead of the upregulation upon infection of IAPV, and silencing of *Vago* upon IAPV infection leads to downregulation of *Dicer-2*. Thus, we propose a more complex communication of the siRNA pathway and the JAK/STAT pathway through Vago in bumblebee compared with the current reports in insects.

Supplementary data

Table S1: Primers used in this chapter

S1: Conserved proteins sequences used to construct phylogenetic tree of Vago

Table S1: Primers used in this chapter

Name	Primer sequence (5'-3')	Amplicon length	Purpose
qPCRVagoF2	TGTTACCCTTCAACGCAATTC	194	QPCR for Vago
qPCRVagoR2	ACAGATTCCGAAACGCTGAT		
Vago-T7dsF2	TAATACGACTCACTATAGGGAGACCTAGTCCCGGAAGTCGAGA	418	dsRNA synthesis for Vago
Vago-T7dsR2	TAATACGACTCACTATAGGGAGAGTACGTACGAATTACAAGATCAACT		
qPCRHopF3	TGGCACAATGTGTCTCATCTT	186	QPCR for Hop
qPCRHopR3	GAGGTACACAACGAGGTCCAG		
Ds2ndHOP-F4	TAATACGACTCACTATAGGGAGATGTCCTTTGTTTCTGCTCTGGA	374	dsRNA synthesis for Hop
Ds2ndHOP-R4	TAATACGACTCACTATAGGGAGATGACTGTCCTTCAGAATCTTGGA		
q-Ri-dicer2-F2	TGGTCAAAACATCAAGAACAACCA	166	QPCR for Dicer-2
q-Ri-dicer2-R2	GATCGGGGCCATACGAACAT		
C-dsDicer2-2nd-F4	TAATACGACTCACTATAGGGAGAGCGAAGGTGTCACCAAATGT	437	dsRNA synthesis for Dicer-2
C-dsDicer2-2nd-R4	TAATACGACTCACTATAGGGAGAGGGTGTGTAAAGGCCTGCAA		

S1: Conserved proteins sequences used to construct phylogenetic tree of Vago

>Bombus_terrestris

MKLVPVLLLVAAIVFAAEEKKEEERPKTFRRLIPADVLRDFPGMCFASTRCATIEPTKSWDLTPFCGRSTCV
PADDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTATFPDCCPIFECEEGAKLEYPEIPTLPPPTEIVETEKTPEATPA

>PREDICTED:_uncharacterized_protein_LOC411622_Apis_mellifera.

MKFAPILLFVIAIVFAAEE-
KEEERPKTFRRLIPADVLRDFPGMCFASTRCATIEPTKSWELTPFCGRSTCVPADDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTAAFPNCCPIFECEEGAKLEYPEIPTLPPPTEIIETEKTSEEVPT

>PREDICTED:_uncharacterized_protein_LOC105185794_Harpegnathos_saltator.

MEHAFVLLFTTIVFIAIADEKEEERPKTFRRLIPADVLRDFPGMCFASTKCATIEPTKTWELSPFCGRSTCVPA
DDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTASFPDCCPIFECEDGAKLEYPEIPTLPPPTEIEASEITAAPKV-

>PREDICTED:_uncharacterized_protein_LOC100878806_Megachile_rotundata.

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KEEERPKTFRRLIPADVLRDFPGRCFASTKCATIEPTKSWDLTPFCGRSTCVPADDNSGR

LFELVEDCGPLPKANPKCKLSEKTNKSAPFPDCCPIFECEDGAKLEYPEIPTLPPPTEIVETEKTPEAAPA

>PREDICTED:_uncharacterized_protein_LOC105623255_Atta_cephalotes.

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VPADDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTASFPECCPIFECEDGAKLEYPEIPTLPPPSDEDAAKAQPETPKP

>PREDICTED:_uncharacterized_protein_LOC105282831_isoform_X2_Cerapachys_biroi.

MAFLLPHPCASSSV---------KIERNFR--------ENFPGMCFASTKCATIEPTKSWQLAPFCGRSTCVPADDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTASFPDCCPIFECEDGAKLEYPEIPTLPTPTED-AAKGEPAAAAP

>PREDICTED:_uncharacterized_protein_LOC105148313_Acromyrmex_echinatior.

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VPADDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTASFPECCPIFECENGAKLEYPEIPTLPPPSED-AAKAQPEAPKP

>PREDICTED:_uncharacterized_protein_LOC105455873_Wasmannia_auropunctata.

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LFELVEDCGPLPKANPKCKLSDKTNKTSSFPDCCPIFECEDGAKLEYPEIPTLPPPSGEDAAAAQPQAPKP

>PREDICTED:_uncharacterized_protein_LOC105258902_Camponotus_floridanus.

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ADDNSGR

LFELVEDCGPLPKANPKCKLSEKTNKTASFPDCCPIFECEEGVKLEYPEIPTLPPPTEAEAANVPPEATPK

>PREDICTED:_uncharacterized_protein_LOC105561162_Vollenhovia_emeryi.

MKTIFVLLCVAVAFVAAQDVQEQERPKTFRRLIPADVLRDFPGMCFASTKCATIEPTKSWELAPFCGRSTC
VPADDNSGR

LFELVEDCGPLPKANSKCKLSDKTNKTASFPDCCPIFECEDGAKLEYPDIPTLPPPSDAI-AKAQPETPKP

>PREDICTED:_uncharacterized_protein_LOC103577816_Microplitis_demolitor.

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LFELVEDCGPLPKANPKCKLSEKTNKTASFPDCCPIFECEEGAKLEYPEIPTVPPPPS--EAEAKPEVAKV

>PREDICTED:_uncharacterized_protein_LOC105197444_Solenopsis_invicta.

MKI-VFALCVAVAFVAANA-
EEQERPKTFRRLIPADVLRDFPGMCFASTKCATIEPLKSWELSPFCGRSTCVLADDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTASFPDCCPLFECEEGAKLEYPEIPTLPPPSGVDTAKAQPEAPQS

>PREDICTED:_uncharacterized_protein_LOC100113619_Nasonia_vitripennis.

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VPADDNSGR

LFELVEDCGPLPKANPKCKLSEKTNKTAPFPDCCPVFECEDGAKLEYPEIPTLPPPTAEEIAKAAAAAGKPA

>PREDICTED:_LOW_QUALITY_PROTEIN:_uncharacterized_protein_LOC105428984_partial_Pogonomyrmex_
barbatus.

WKLTYLFTISEVLFFDSFLSGFYPNKRKCHLFVLFHYIVDFPGTCFASTKCATIEPGKSWELSPFCGRSTCVS
ADDNSGR

LFELVEDCGPLPKANPKCKLSDKTNKTASFPECCPIFECEDGATLEYPDIPTLPPPTED-AAKAQPEAPKP

>PREDICTED:_uncharacterized_protein_LOC105268204_Fopius_arisanus.

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>conserved_hypothetical_protein_Pediculus_humanus_corporis.

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VAEEQAGR

LLELVEDCGPLPKANPKCKLSEKTNKTAPFPDCCPIFECEKGVKLEYPELPTVPPPTDKKKA---------

>CG31997_isoform_A_Drosophila_melanogaster.

MSFHTLILTAFTVSLCAEQKKSDAGERIFKRLIPADVLRDFPGMCFASTRCATVEPGKSWDLTPFCGRSTCV
QNEENDAK

LFELVEDCGPLPLANDKCKLTEKTNKTASFPYCCPIFTCDPGVKLEYPEIGKDNDKKNSE-----------

>PREDICTED:_uncharacterized_protein_LOC105220319_Bactrocera_cucurbitae.

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VQNDENPAK

LLELVEDCGPLPLANEKCKLTEKTNKTAPFPYCCPIFTCEPGVKLEYPEIPKEEAKKE----------

>uncharacterized_protein_LOC100167365_precursor_Acyrthosiphon_pisum.

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VQGE-GTDR

LLELVEDCGPYPKSNPKCKLSEKTNKTASFPECCPVFDCEPGVKLEYPEVTVVENPESSDDSTTTTSTEKP

>PREDICTED:_uncharacterized_protein_LOC655864_Tribolium_castaneum.

MKV-
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DKPPR

LLELVEDCGPLPLANPKCKLEEKTNKTAAFPDCCPVFKCEEGAKLEYPEIPTVAPVPE--ASSTTPKA---

>hypothetical_protein_KGM_07285_Danaus_plexippus.

MKY-LIVLAIIAFSYAADD-
KDDERPKTFRRLIPADVLRDFPGLCFASTRCATVEPGNTWDLAPFCGKSTCVVSEDTPPR

LLELVEDCGPLPIANPKCKLTDKTNKTAPFPDCCPIFTCEDGVKLEYPELPTPPEEEKKEKS---------

>PREDICTED:_uncharacterized_protein_LOC105665112_Ceratitis_capitata.

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VQNEDNPSK

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>unknown_Manduca_sexta.

MKYLVVVAVFALAFAAEEKE---
DIPKTYKRLIPADVLRDFPGTCFASTRCATVEPGNTWELSPFCGRSTCVLSEDHPPR

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>similar_to_CG31997_Papilio_xuthus.

MKYLFVLAVFALAFAAEEE-
KDGERPKTFRRLIPADVLRDFPGLCFASTRCATVEPGNSWDLAPFCGRSMCVVSEDTPPR

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>conserved_hypothetical_protein_Culex_quinquefasciatus.

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VVSESNPAQ

LLELVEDCGPLPLANDKCKLTDKTNKTAPFPYCCPKFTCEPGVKLEYPEIKPSDASEEKK----------

>AAEL000445-PA_Aedes_aegypti.

GVLIAATLCVANVFAADEAPTDEDTVKIYKRLIPADVLRDFPGMCFASTRCATIEPGKSWDLAPFCGRSTCV
VSETNPSQ

LLELVEDCGPLPLANDKCKLTDKTNKTAPFPYCCPKFTCEPGVKLEYPDVKGPEAAPEDD--KKN------

>PREDICTED:_uncharacterized_protein_LOC105381502_Plutella_xylostella.

MKY-
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DEPPR

LLELVEDCGPLPLANDKCKLTDKTNKTAPFPGCCPVFTCTDGAKLEYPELPTPPPEGE----EKKPEEEKP

>PREDICTED:_uncharacterized_protein_LOC101888253_Musca_domestica.

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VQNEEDPSK

LLELVEDCGPLPLANDKCKLTEKTNKTAAFPYCCPVFTCEPGVKLEYPEAVKETKKE----------

>AGAP002085-PA_Anopheles_gambiae_str._PEST.

MRSFTVVAVLALYVQAADEATKDAAPKTYKRLIPADVLRDFPGMCFASTKCATFEPGQYWDLTPFCGRST
CVLSDDAQPR

LLELVEDCGPLPLANDKCKLTEKTNKTAPFPACCPTFTCEPGAKLEYPEIKTAPESTSEQN----------

>hypothetical_protein_YQE_10125_partial_Dendroctonus_ponderosae.

----------MCFASTKCATVEPGKTWELYPFCGRSTCVVSEDQPPR

LLELVEDCGPLPLANEKCKLEEKTNKTAPFPACCPEFKCEAGAKLEYPEIPTVAPVPEDASTTTAKSA---

>PREDICTED:_uncharacterized_protein_LOC101741978_Bombyx_mori.

MKYLIVLAVFALAFAAEEKE---
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LLELVEDCGPLPLTNPKCQLTEKTNKTAPFPGCCPIFTCEEGAKLEYPELPTPPPEDK--KAEEKPKA---

>unknown_Culicoides_sp._LJH-2002.

MRFPFVFFVIVSLSFASET-
KNDDGIKVYKRLIPADVLRDFPGVCFASTKCAMFEPGKQWDLKPFCGRSTCVTPEDGSSR

LLELVEDCGPLPIANDKCKLTEKTNKTADFPYCCPQFACQDGAKLEYPEVKTSSA----------

>hypothetical_protein_DAPPUDRAFT_214640_Daphnia_pulex.

MKFILCFSLLFVLVASVE-----ITPKTLTREIKADVLRDFPGVCYASTQCRTFKENEEWDLKPFCGKSICIKGA--DGI

LKERVSDCGPPAKANPECKV--NANATLPYPNCCPVYDCAPGVQLEFPDIPVA----------

>single_VWC_domain_protein_1_Litopenaeus_vannamei.

MKF-
LLIACLGSLVFAQQGPADLQGPGPFVRDLKADVLRDFPELCFSSTNFRLFLENQSWSLFPFCGKAECVKSG---AD

YIERVHDCGPQPKNAEACTILQRNDTILEYPSCCPKYVCPDGVTLEYPEIQKQNQAALQAAAAAREAAGPQ

>Chain_A_Structure_Of_A_Chordin-Like_Cysteine-Rich_Repeat_(Vwc_Module)_From_Collagen_Iia.

----------YVEFQEA----------GSCVQDGQ--RYNDKDVWKPEP-CRICVCDTGTLCDDI

ICEDVKDC----------LSPEIPFGECCPI--CPA-------DLAAAA----------

Chapter V -General conclusions and future perspectives

1. General conclusions

The involvement of siRNA, miRNA, and JAK/STAT pathways upon viral infections, were concluded, respectively, in Chapter 3, 4 and 5. Some key points are listed here:

1.1. The siRNA pathway upon viral infections

Although the siRNA pathway is the most studied pathway in bee virus research and its usefulness to tackle bee virus-related diseases in the field is promising, the involvement of the siRNA pathway in the defense against different bee viruses is still poorly understood. Our results showed that the infections of IAPV and SBPV could both induce the expression of *Dicer-2*, and IAPV infections triggered the production of predominantly ~22 nt IAPV-derived siRNAs with a strong antigenome polarity whereas no Dicer-2-processed ~22 nt vsiRNA were detected in SBPV infection. Together with the "RNAi of RNAi" experiment on *Dicer-2* which did not result in altered genome copy numbers of IAPV or SBPV, we speculated about the importance of the siRNA pathway as an antiviral response against bee viruses. For IAPV, this pathway was recruited but was insufficient to control viral infection. While SBPV seemed to be controlled by the host, aside from *Dicer-2* induction upon viral infection, no evidence of the activity of the siRNA pathway was found, suggesting that other pathway with antiviral activity may play more important roles in managing viral infections.

1.2. The miRNA pathway upon viral infections

The miRNA pathway, a closely related pathway to the siRNA pathway, is also established to be involved in insect-pathogen interactions. In a virus-infected host the miRNAs can have two origins, either encoded by the host or the virus. Both origins of miRNAs may target host genes and/or the viral RNA (genome), which results in a complex network of host-virus interactions based on miRNAs. Our results showed that the infections of IAPV and SBPV could alter the expressions of *Dicer-1* and/or *Ago-1* and the expression of some different miRNAs, which might indicate the involvement of the miRNA pathway in bumblebee-virus interaction. The targets predicted for these differentially expressed miRNAs clustered into some bumblebee Gene Ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, which may represent a possible regulatory network in virus-host interaction. Intriguingly, the target predictions showed that these differentially expressed host miRNAs could also directly target the viral RNA (genome). The silencing of *Dicer-1* did not lead to an altered genome copy number of SBPV, which might imply that these differentially expressed bumblebee miRNAs could serve as co-interests for the host and the virus, and/or non-canonical production of miRNAs may also play an important role.

1.3. The JAK/STAT pathway upon viral infections

Next to the nucleotide-based RNAi (siRNA and miRNA pathways) immunity, there exists another conserved protein-based pathway in insects, JAK/STAT, directly contributes to combat viral infections. In addition, it also represents a cross-talk with the siRNA pathway through Vago. Our results showed that the infections of IAPV and SBPV did not result in any detected effect in the expression of *Hop*, but the silencing of *Hop* through RNAi resulted in an increased gcn for

SBPV but not for IAPV. These results suggested a role of the bumblebee JAK/STAT pathway in the control of an avirulent virus SBPV, but its role in the defense against a virulent virus as IAPV remained unclear. The potent cross-talk of the siRNA and JAK/STAT pathways through Vago might be occurring differently in the bumblebee compared with that in the mosquito. Here, we proposed a more complex communication between the siRNA pathway and the JAK/STAT pathway through Vago in bumblebees compared with the current reports in insects.

2. Interactions of the siRNA, miRNA, and JAK/STAT pathways upon viral infections

The siRNA, miRNA and JAK/STAT pathways may not act linearly but may also communicate with each other. With some initial evidences, we proposed possible interplay among them. We also speculated some possible future research directions to see if these required fundamental knowledges could be valorized in actual applications to improve bee health.

2.1. Interaction between the siRNA pathway and the miRNA pathway upon viral infections

In insects, the siRNA and miRNA pathways are both essentially evolved to regulate gene expressions. Although each pathway has its own machinery to regulate gene regulation, some core components, such as Ago-2 are shared by both pathways (Chandradoss et al., 2015). In our study, we showed some evidences on the involvement of both pathways upon viral infections in bumblebees (Chapter III and IV). In addition, our small RNAs sequencing not only revealed the

virus-derived siRNAs, especially 22nt vsiRNAs as the possibly processing activity of Dicer-2, but also some peaks of small RNAs which may have possible functions, such as viral originated miRNAs. In fact, virus-encoded miRNAs could serve different interests to host and virus (Asgari, 2015). Virus-encoded miRNAs could manipulate the host machinery to benefit for its own pathogenicity (Singh et al., 2012). Virus can also negatively regulate their own replication by encoding miRNA (Hussain and Asgari, 2014a). Another interesting observation from our study was that the large fraction of miRNAs with differential expressions were predicted to potentially target viral genomes in bumblebees, which certainly needs further investigation before drawing any conclusions.

Currently there is a focus on virus-specific dsRNA to trigger the siRNAs pathway. This approach has been proven to be potent to control bee viruses (Niu et al., 2014b). Indeed the pioneer company Beeologics is currently working on the BioDirect™ technology, a bee health product based on dsRNA molecules to control bee viruses and *Varroa* mites. This strategy is only based on antiviral response from the siRNA pathway, but another closed related pathway, the miRNA pathway, could also be promising in control viruses as well. Elaborating on potential interactions of two small non-coding RNAs, one could design a new kind of therapeutics to control viral infection based on combinations of the antiviral effect of these two pathways. First of all, more fundamental research is needed to validate candidates of host and virus miRNAs which can target viral genome. In addition, some host miRNAs may also regulate the pathways with antiviral activities which could also be evaluated. Secondly, through the use of mimics (gain of function) or antagomirs (loss of function) of candidate miRNAs, we can identify good candidates of miRNAs which have an inhibiting effect on viral infection. Furthermore, a design of mixing the virus-specific dsRNA/siRNA and miRNA which can directly target the viral genome, may be

more effective and also be difficult for virus to develop resistance mechanisms as taking advantage of two RNAi pathways (siRNA and miRNA).

2.2. Interaction between the siRNA pathway and the JAK/STAT pathway upon viral infections

In both honeybees and bumblebees non-specific dsRNA can induce a noticeable host antiviral activity (Flenniken and Andino, 2013; Piot et al., 2015). Based on the current understanding, two interpretations can be associated: one is that dsRNA could act as a PAMP which can induce several immune pathways, instead of only the siRNA pathway. The investigation on the majority of canonical insect immune pathways in honeybee upon viral infections could not link any of these with the antiviral activity of the non-specific dsRNA molecule induced (Flenniken and Andino, 2013). This study may imply that some unknown pathways are holding keys in non-specific dsRNA induced antiviral immunity in bees. The other is that non-specific dsRNA can activate the siRNA pathway, and this pathway communicates with other immune pathways in order to achieve effective immune responses. The latter seems to fit with our observation on interactions of the siRNA and JAK/STAT pathways through Vago in bumblebee upon viral infection. In addition, this interaction in bumblebee seemed more complex than that in the well-described cross-talk of these two pathways in mosquito. Therefore, it is worthwhile to focus on the validation of two key communication genes, *Dicer-2* and *Vago*, which could be good targets to enhance the bees' antiviral activity. This is still very speculative but enhancing the activity of either gene could be a potential direction for application-based research. For instance, non-specific dsRNA as well as some avirulent virus such as SBPV can induce the expression of *Dicer-2* thus may enhance the siRNA antiviral response and/or leading a further communication with another immune pathway. This also shows the idea of using an avirulent virus to combat

some virulent virus. Another approach is to make a Vago-associated vaccine to control viruses in beneficial insects.

2.3. Interaction between the miRNA pathway and the JAK/STAT pathway upon viral infections

Several differentially expressed bumblebee miRNAs upon infections of IAPV and/or SBPV, are predicted to possibly regulate some immune pathways, such as JAK/STAT pathway (KEGG:04630), Hippo signaling pathway (KEGG:04390), and Phagosome (KEGG:04145). This initial result requires more studies to investigate the miRNAs in the interaction with immune pathways in general, specially, the JAK/STAT pathway. Our recommended approach would be firstly to validate the miRNAs in the regulations with the immune genes. On the next level we proposed to test good candidate miRNAs mimics (gain of function) or antagomirs (loss of function) which could enhance host immune activities and lead to an inhibition on viral infection.

In summary these ideas presented above allow us to explore a small RNAs-based approach, which was not only focused on direct degradation of viral genome (RNA), but also focus on enhancing bee antiviral immunities.

3. Some points to rethink about host-virus interaction

3.1. Not only proteins but also non-coding RNAs

As an obligated parasite of living cells, virus is dependent on the host machinery to achieve its own replication. Thus two factors should always be kept in mind, the virus and the host, when

thinking about viral pathogenicity. Currently, proteins are generally recognized as the main functional building blocks of life, however, with more and more increasing knowledge, it seems that non-coding RNAs (ncRNAs) play an important role in gene regulation by interfering protein translation (Cech and Steitz, 2014). Especially, in the case of host-virus interaction, the ncRNAs-based regulation tends to be more straightforward between host-virus compared with protein regulation as both players can easily take advantage of the ncRNAs. Therefore, both proteins and ncRNAs should be investigated to study the regulation on host-virus interaction.

3.2. Host developmental stages

It is not only the viruses' characters which can determine differences in host antiviral immune responses, the insect host developmental stages are also important. In our current study, we only tested antiviral immunity in the adult stage, which is the stage that goes out to forage and might meet potential viruses. On the other hand, viral transmission inside colonies is also regularly happening. Some viral infections actually only occur in larval stages through ingestion of the collected foods containing virions. However, a study on immature bee stages is difficult, especially, in bumblebees. *In vitro* cultivation of larva is still not possible thus all experiments need to be done within the colony. The experimentally treated larvae are often not accepted by the colony again, thus the current methodology still does not allow a feasible setup when trying to work with larvae.

Another aspect which needs to be considered is that studies are mostly performed on the non-fertile workers, as these are produced in mass. Bumblebees are social insects and the health of queen is critical to maintain the colony, thus the study on the queen in combating viral infections is also needed to understand the antiviral immunity of bees.

3.3. Multiple viral infections

Multiple viral infections (De Smet et al., 2012; McMahon et al., 2015; Singh et al., 2010) can be an important context in impacting bee antiviral immunity. Generally, bee viruses and their hosts form a complex network, where multiple viruses can infect a single host. How one viral infection can influence the infection of the other viruses still remains unknown. This kind of interaction is indirect, which could be linked into two ways. One is that a viral infection may trigger host immunity which may impact on the latter viral invasion, if the first viral infection could induce an antiviral immunity and may make the host immune system ready to combat the latter viral infection. The second pattern could be that the first viral infection inhibits the host antiviral immunity, thus it would give the latter viral infection some advantage to get a successful invasion. For example, some viruses can encode VSR to compete with the immune activity from RNAi. It is not unlikely that some viruses benefit from VSR encoded by co-infected viruses.

3.4. From lab to field: consider more factors

In nature, various factors can have an impact on the host antiviral immunity, while immunological studies often remain within the context of the lab where we have control over external conditions and can study the exact "point to point" interaction. The study in non-model but economically important insects is often not only to understand their immunity but also to try to find the solutions, such as for bee decline in an association with viruses. Wild bees in nature all encounter many factors that could influence their antiviral immunity, the well described factors such as *Varroa* mites (McMenamin and Genersch, 2015), pesticides (Di Prisco et al., 2013) and the overwinter period (Steinmann et al., 2015), can influence viral infection patterns. Considering the complexity of nature, these factors are still too few to provide the picture of the real bee-virus interaction in the field.

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Summary

Understanding the interaction between host and virus, especially host antiviral immunity in super model insects such as *Drosophila* and mosquitoes, greatly facilitates the research in non-model insects. Although honeybees, bumblebees and solitary bees show behavior ranging from eusocial to solitary, genome-level comparison shows they all have a relatively small immune repertoire compared with other insects. This makes the study of immunity in these bees needed to not only understand insect immune evolution, but also to elucidate hos different they are from those super model insects and if general knowledges could be expanded by different organisms. Here in this project we worked with the bumblebee, *Bombus terrestris*, which stands in an intermediate position on the eusocial spectrum. Currently, scientists are trying to understand the reasons for bee decline in the association with different kinds of viral infections. Thus an exact antiviral immunity study in the bee itself in correspondence to different bee viruses is important to draw the correct conclusions and to search for efficient measures to tackle the current virus-related problem in bees. We initiated this study by optimizing the reference genes to perform RT-qPCR analyses for viral detection and gene expression measurements and also the conditions to obtain a sufficient RNAi efficacy for gene silencing. Both are two key strategies used in this dissertation. As the innate immunity in insects is not a static response, it could be possibly altered by a different virulence of viral infections. Here, we opted for two viruses, the Israeli acute paralysis virus (IAPV) and the slow bee paralysis virus (SBPV), and this decision was based on: 1) both viruses naturally infect bumblebees in the field; 2) each represents a key bee virus family, i.e. *Dicistroviridae* and *Iflaviridae*; 3) in our experimental setup, IAPV (as virulent infection) induces an extremely virulent infection while SBPV (as avirulent infection) does not cause mortality of bees. On the basis of the current understanding of antiviral immunity of bees, we chose three important and possibly interacting pathways, namely the small interfering RNA (siRNA), microRNA (miRNA) and JAK/STAT pathways, to investigate the immune responses of bumblebees in their defense against viral infections.

The siRNA pathway activity upon viral infections

Although the siRNA pathway is the most studied pathway with antiviral activity in bee virus research and its usefulness to tackle bee virus-related diseases in the field is promising, the involvement of the siRNA pathway in the defense against different bee viruses is still poorly understood. Our results showed that the infections of IAPV and SBPV could both induce the expression of *Dicer-2*, and IAPV infections also triggered the production of predominantly ~22 nt IAPV-derived siRNAs (vsiRNAs) with a strong antigenome polarity whereas no Dicer-2-processed ~22 nt vsiRNA were detected in SBPV infection. Together with the "RNAi of RNAi" experiment on *Dicer-2* which did not result in altered genome copy numbers of IAPV or SBPV, we can speculate about the importance of the siRNA pathway as an antiviral response against bee viruses. For IAPV, this pathway is recruited but is insufficient to control viral infection. While SBPV seems to be controlled by the host, aside from *Dicer-2* induction upon viral infection, no evidence of the activity of the siRNA pathway was found, suggesting other pathway with antiviral activity may play more important roles.

The miRNA pathway activity upon viral infections

The miRNA pathway, as a closely related pathway to the siRNA pathway, is also established to be involved in insect-pathogen interactions. In a virus-infected host the miRNAs can have two origins, either encoded by the host or the virus. Both origins of miRNAs may target host genes and/or the viral RNA (genome), which results in a complex network of host-virus interactions based on miRNAs. Our results showed that the infections of IAPV and SBPV could alter the expressions of *Dicer-1* and/or *Ago-1* and the expression of multiple miRNAs, which might indicate the involvement of the miRNA pathway in bumblebee-virus interaction. The targets predicted for these differentially expressed miRNAs clustered into some bumblebee Gene Ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, which may represent a possible regulatory network. Intriguingly, the prediction showed that some differentially expressed host miRNAs could also directly target the viral RNA (genome). The silencing of *Dicer-1* did not lead to an altered genome copy number of SBPV, which might imply that these differentially expressed bumblebee miRNAs could serve as co-interests for the host and the virus, and/or non-canonical production of miRNAs may also play an important role.

The JAK/STAT pathway activity upon viral infections

Next to the nucleotide-based RNAi (siRNA and miRNA pathways) immunity, there exists another conserved protein-based pathway in insects, JAK/STAT directly contributes to deal with viral infections. It also shows a cross-talk with the siRNA pathway upon viral infections to rise efficient antiviral response. Our results showed that the infections of IAPV and SBPV did not result in any detected effect in the expression of *Hop*, but the silencing of *Hop* through RNAi resulted in an increased genome copy number for SBPV but not for IAPV. These results suggest a role of the bumblebee JAK/STAT pathway in the control of an avirulent virus SBPV, but its role in the defense against a virulent virus as IAPV remains unclear. The potent cross-talk of the siRNA and JAK/STAT pathways through Vago might be occurring differently in the bumblebee compared with the mosquito. Thus, we propose a more complex communication between the siRNA pathway and the JAK/STAT pathway through Vago in bumblebees compared with the current reports in insects.

Samenvatting

Onze kennis omtrent de interactie tussen virussen en hun gastheer, meer bepaald over de antivirale immuniteit in modelinsecten zoals *Drosophila* en de mug vereenvoudigt gelijkaardig onderzoek in niet-model insecten. Alhoewel honingbijen, hommels en solitaire bijen een gedrag vertonen dat reikt van een eusociaal tot solitair bestaan, toch werd tijdens het vergelijken van hun genomen gevonden dat ze allen slechts over een relatief klein aantal genen beschikken die betrokken zijn in immuniteit, in vergelijking met andere insecten. Hierdoor is de studie van immuniteit in deze bijen waardevol om zo de evolutie van immuniteit in insecten te doorgronden. In dit onderzoek werd gewerkt met de aardhommel, *Bombus terrestris*, die een gedrag vertoont dat intermediair is tussen eusociaal en solitair. Op dit moment trachten wetenschappers de redenen voor de achteruitgang van bijen te doorgronden, onder andere de rol die de verschillende soorten virussen hierin kunnen spelen. Daarom is de studie van antivirale immuniteit ten gevolge van verschillende types van virussen in de bij belangrijk om zo de juiste conclusies te kunnen trekken en op zoek te gaan naar efficiënte methoden om de virus-gerelateerde problemen in bijen aan te pakken.

In een eerste fase van deze studie werden twee belangrijke strategieën binnen dit werk geoptimaliseerd: als eerste werden geschikte referentiegenen voor RT-qPCR bepaald om zo een betrouwbare virale detectie en meting van genexpressie mogelijk te maken, en daarnaast werden ook de parameters voor het bereiken van een succesvol RNAi experiment geoptimaliseerd. Aangezien de aangeboren immuunrespons in insecten niet statisch is, zou deze mogelijk afhankelijk kunnen zijn van verschillen in virulentie tussen virale infecties. Er werd gekozen te

werken met twee virussen: Israeli acute paralysis virus (IAPV) en slow bee paralysis virus (SBPV). Deze beslissing was gebaseerd op drie observaties: 1) beide kunnen op een natuurlijke wijze hommels infecteren in het veld, 2) ze vertegenwoordigen elk een belangrijke bijenvirus-familie, i.e. de *Dicistroviridae* en *Iflaviridae*, en 3) tijdens experimenten werd gezien dat IAPV een zeer virulente infectie veroorzaakt, terwijl een SBPV-infectie niet tot mortaliteit leidt. Op basis van wat op dit moment geweten is over antivirale immuniteit in bijen werden drie interessante pathways geselecteerd (die mogelijk ook met elkaar interageren): de small interfering RNA (siRNA), microRNA (miRNA) en JAK/STAT pathway, om zo de immuunrespons tegen virale infecties in hommels te onderzoeken.

De activiteit van de siRNA pathway bij virale infecties

Alhoewel de siRNA pathway de meest bestudeerde antivirale pathway in bijen is en het een zeer beloftevolle techniek lijkt om bijenvirussen te bestrijden in het veld, is nog steeds weinig geweten over de betrokkenheid van deze pathway in de afweer tegen verschillende bijenvirussen. Onze resultaten tonen dat infecties door beiden IAPV en SBPV de expressie van *Dicer-2* induceren en dat IAPV-infecties leiden tot de productie van voornamelijk 22nt-lange siRNAs afgeleid van IAPV (vsiRNAs). Deze vertonen een sterke polariteit voor het antigenoom. In het geval van een SBPV-infectie werden geen 22nt-lange vsiRNAs, geproduceerd door Dicer-2, gedetecteerd. Wanneer we ook het "RNAi of RNAi" experiment in rekening brengen waarin het silencen van *Dicer-2* niet resulteerde in veranderingen in aantal genoomkopijen van IAPV of SBPV, kunnen we speculeren over het belang van de siRNA pathway in de antivirale immuunrespons tegen bijenvirussen. In het geval van IAPV is de pathway wel geactiveerd maar is ze onvoldoende om de virale infectie in te perken. Bij SBPV-infectie slaagt de gastheer er wel in om de infectie van SBPV te controleren. Maar in dit geval werd behalve de inductie van

Dicer-2 geen bewijs gevonden van de activiteit van de siRNA pathway, wat doet vermoeden dat andere antivirale pathways een belangrijkere rol spelen

De activiteit van de miRNA pathway bij virale infecties

De miRNA pathway is nauw verwant met de siRNA pathway en zijn betrokkenheid bij insect-pathogeen interacties werd reeds gerapporteerd. In een virus-geïnfecteerde gastheer kunnen miRNAs ontstaan op twee manieren: gecodeerd door de gastheer of door het virus. Beide types van miRNAs kunnen genen van de gastheer en/of het virus als doelwit hebben, wat leidt tot een complex netwerk van gastheer-virus interacties. Onze resultaten toonden dat infecties met IAPV en SBPV de expressie van *Dicer-1* en *Ago-1* konden veranderen. Daarnaast werden meerdere miRNAs met een veranderd expressieniveau gevonden, wat erop wijst dat de miRNA pathway mogelijk betrokken is in de interactie tussen de hommel en het virus. Voor de miRNAs die verschillen in expressie vertoonden werden de targets voorspeld en deze clusterden in enkele Gene Ontology (GO) termen of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, die een regulerend netwerk vormen. Intrigerend is dat deze gastheer-miRNAs volgens onze predictie ook het virale RNA (genoom) kunnen targetten. Het silencen van *Dicer-1* zorgde niet voor een veranderd aantal genoomkopijen van SBPV. Dit wijst erop dat deze miRNAs, die verschillend tot expressie komen na virusinfectie in de hommel, voordelig kunnen zijn voor zowel de gastheer als het virus en dat de niet-gebruikelijke productie van miRNAs ook een belangrijke rol kan spelen.

De activiteit van de JAK/STAT pathway bij virale infecties

Naast de RNAi (zowel siRNA als miRNA pathways)-gebaseerde immuniteit, op nucleotideniveau, is er een andere geconserveerde, op eiwit gebaseerde pathway beschikbaar in

insecten, namelijk de JAK/STAT pathway. Deze vertoont niet alleen antivirale activiteit, maar ook crosstalk met de siRNA pathway. Onze resultaten toonden aan dat infecties met IAPV en SBPV geen veranderingen in expressie van *Hop* teweeg brachten, en dat het silencen van *Hop* wel een verhoogd aantal genoomkopijen oplevert bij SBPV, maar niet bij IAPV. Dit doet vermoeden dat de JAK/STAT pathway een rol speelt in de afweer tegen het avirulent virus SBPV, maar zijn betrokkenheid in de afweer tegen het virulent virus IAPV blijft onbekend. De krachtige crosstalk tussen de siRNA pathway en de JAK/STAT pathway met behulp van Vago kan mogelijk op een andere manier gebeuren in de hommel dan in de mug. We suggereren dan ook dat er een meer complexe communicatie aan de gang is tussen de siRNA pathway en de JAK/STAT pathway met behulp van Vago in de hommel dan wat tot nu toe beschreven is in insecten.

Curriculum Vitae

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Publications associated with this project

Niu, J, Meeus I, Smagghe G, 2015. Involvement of *Bombus terrestris* JAK/STAT pathway in antiviral response and possible interaction with the small interfering RNA pathway through Vago. Scientific Reports submitted.

Niu J, Smagghe G, Coninck DID, Nieuwerburgh FV, Deforce D, Meeus I. (2015) In vivo study of *Dicer-2* mediated immune response from the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in *Bombus terrestris*. Insect Biochemistry and Molecular Biology Submitted.

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Conferences with oral presentation

08/2015: 48th Annual Meeting of the Society for Invertebrate Pathology, Vancouver, British Columbia, Canada Presentation title: From the mosquito model to the bumblebee: a different behaviour of Vago mediated cross-talk between the small interfering RNA and JAK/STAT pathways upon virus infection. (**FWO travel grant**)

08/2014: 47th Annual Meeting of the Society for Invertebrate Pathology, Mainz, Germany. Presentation title: The involvement of bumblebee small interfering RNA pathway against two different bee viruses.

08/2013: 46th Annual Meeting of the Society for Invertebrate Pathology, Pittsburgh, Pennsylvania, USA Presentation title: Study on interaction between bumblebee *Bombus terrestris* RNAi pathway and IAPV/KBV by RT-qPCR. (**FWO travel grant**)

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My Belgium friends and housemates: I admired all your effort to constrain yourself not to speak Dutch when I was present. I am sorry I tried my best to learn your language but it did not work out…I learned lots of Belgium culture from you and most of them were very interesting for me. I had lots of happy moments with you.

To my beloved family（致我的家人）:

感谢你们这么多年对我的梦想的支持，感谢你们的辛勤付出。

Jinzhi Niu（牛金志）

September 17, 2015