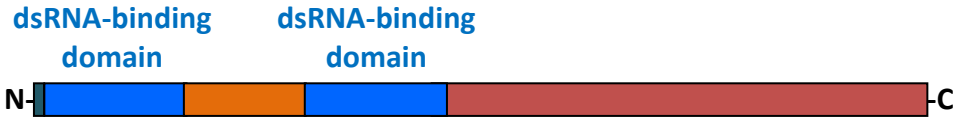
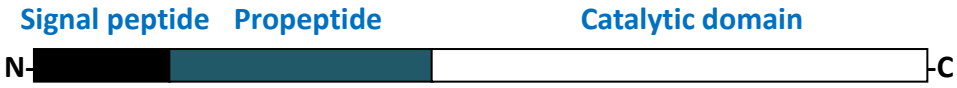
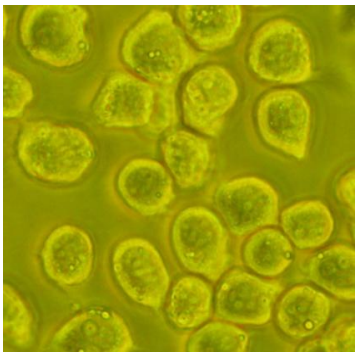
BmR2D2



BmdsRNase



BmToll9-1



Interactions between dsRNA and factors affecting the efficiency of RNAi in silkmoth (*Bombyx mori*)

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UNIVERSITEIT GENT

FACULTY OF BIOSCIENCE ENGINEERING

Life is like a roller coaster. Sometimes it goes up and sometimes it goes down.

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Interactions between dsRNA and factors affecting the efficiency of RNAi in silkmoth (*Bombyx mori*)

Jisheng Liu

Thesis submitted in fulfillment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:
Interacties tussen dsRNA en factoren die de efficiëntie van RNAi beïnvloeden bij de zijderups (*Bombyx mori*)

Front cover: Top: the protein structure of BmR2D2, BmdsRNase and BmToll9-1. Bottom left: the silkmoth larva at 5^{th} instar. Bottome right: the silkmoth ovaries-derived Bm5 cells.

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List of abbreviations

Ago2	Argonaute-2
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
Att	Attacin
C3PO	Component 3 promoter of RISC
cDNA	Complementary DNA
CecE	Cecropin E
CPV	Cytoplasmic polyhedrosis virus
Cq	Quantification cycle
CrPV	Cricket paralysis virus
DAP	Diaminopimelic
Dcr2	Dicer-2
DCV	*Drosophila* C virus
Def	Defensin
dFADD	*Drosophila* Fas-associated death domain
Dif	Dorsal-related immunity factor
Dome	Domeless
DREDD	Death related ced-3/Nedd2-like protein
dsRNA	Double-stranded RNA
dsRNase	Double-stranded ribonuclease
DXV	*Drosophila* X virus
ECD	Ectodomain
endo-siRNA	Endogenous siRNA
ERI	Enhanced RNAi
esiRNA	Endogenous siRNA
EST	Expressed sequence tag
FBS	Fetal bovine serum
FHV	Flock house virus
GFP	Green fluorescent protein
GST	Glutathione-*S*-transferase
Hem	Hemolin

Hop	Hopscotch
IKK	Iκb kinase
IL-1	Interleukin 1
IMD	Immune deficiency
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Leb	Lebocin
Loqs	Loquacious
LPS	Lipopolysaccharide
Lys	Lysozyme
miRNA	MicroRNA
Mor	Moricin
MyD88	Myeloid differentiation factor 88
NPV	Nuclear polyhedrosis virus
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PGRP-LC	Peptidoglycan recognition protein LC
PGRP-SA	Peptidoglycan recognition receptor SA
PI3K	Phosphatidylinositol 3-kinase
piRNA	PIWI-interacting RNA
PRR	Pattern recognition receptor
qPCR	Quantitative real-time polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RAPD	Random amplified polymorphic DNA
RdRP	RNA-dependent RNA polymerase
RFLP	Restriction fragment length polymorphic
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase	Ribonuclease

RNase III	Ribonuclease III
RT-PCR	Reverse-transcription PCR
SINV	Sindbis virus
siRNA	Short interfering RNA
sRNA	Small non-coding RNAs
SSR	Simple sequence repeat
STAT	Signal transducers and activators of transcription
TAB2	TAK1-binding protein 2
TAK1	Transforming growth factor-β-activated kinase 1
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF-R	Tumor necrosis factor-receptor
Tudor-SN	Tudor staphylococcal nuclease
Upd	Unpaired
UTR	Untranslated region
Vir-1	*Virus induced RNA-1*
WGA	Wheat germ agglutinin
WGS	Whole genome shotgun
WNV	West Nile virus
VSR	Viral suppressor of RNA silencing

Chapter 1

General introduction

RNA interference (RNAi) was first discovered in the nematode, *Caenorhabditis elegans* (Fire et al., 1998) which resulted in the Nobel Prize for medicine in 2006. The discovery of RNAi has lead to a revolution in biology (Fire et al., 1998), because a method became available that allowed silencing of gene expression in a wide range of organisms. A key feature of RNAi is the generation of small RNAs consisting of ~20-30 nucleotides which were initially divided into two primary categories: short interfering RNAs (siRNAs) and microRNAs (miRNAs) (Moazed, 2009). More recently, new categories of small RNAs, PIWI-interacting RNAs (piRNAs) and endogenous siRNAs (endo-siRNAs or esiRNAs) were also discovered (Liu et al., 2009; Siomi and Siomi, 2009; Siomi et al., 2010). A common feature of all of these small RNAs is that they are loaded into Argonaute proteins to execute their targeting function. Of all the small RNAs, siRNAs and miRNAs are best known and studied.

The basic RNAi process that involves siRNAs or miRNAs can be divided into three steps. First, a long double-stranded RNA (dsRNA) is processed into small RNA duplexes by a ribonuclease III (RNase III) enzyme known as Dicer. Second, the small RNA duplexes are loaded into a RNA-induced silencing complex (RISC) containing Argonaute proteins as effectors. Third, after unwounding of the small RNA duplex and preserving a single strand, the guide strand, the RISCs actively search target mRNAs and direct silencing by endonuclease cleavage (siRNAs) or by translation inhibition (miRNAs) (Carthew and Sontheimer, 2009).

In the Dipteran insect *Drosophila melanogaster*, the two RNA silencing pathways involving siRNAs and miRNAs are separated with respect to their biogenesis and function. Both types of small RNAs are generated by separate Dicer enzymes: nuclear Drosha and cytoplasmic Dicer-1 for miRNAs and Dicer-2 for siRNAs. Then the duplexes enter RISC assembly pathways with separate Argonaute proteins as core constituents: Ago-1 for miRNAs and Ago-2 for siRNAs (Tomari et al., 2007). The Dicer enzymes generally also function as a complex with dsRNA-binding proteins that are specific to each pathway (Drosha/Pasha and Dicer-1/Loquacious for miRNA pathway and Dicer-2/R2D2 for the siRNA pathway (Carthew and Sontheimer, 2009; Tomari et al., 2007).

However, in another model insect in the insect order of Lepidoptera, *Bombyx mori*, the above two RNA silencing pathways are not as clear as in *Drosophila*. It was also reported that the RNAi efficiency is refractory in this insect order. For *in vivo* applications in insects, the most convenient way to achieve gene silencing would be by injection of dsRNA into the hemolymph of the insect. This approach, in which silencing is achieved in different cells or

tissues after internalization of dsRNA, is called "systemic RNAi" and has led to successful knockdown of the target gene in a considerable number of reports (Mello and Conte, 2004; Posnien et al., 2009; Tomoyasu et al., 2008). While the technique of systemic RNAi is very efficient in Coleopteran insects such as *Tribolium castaneum* (Posnien et al., 2009), a much smaller rate of efficiency has been reported for Lepidopteran insects (Terenius et al., 2011).

The domesticated silkmoth, *Bombyx mori*, has long been used as an important model in Lepidopteran insects, due to its large body size, easy rearing in the laboratory and economic importance in sericulture (Mita et al., 2004). With the development of biotechnology, the publication of silkmoth genome (Mita et al., 2003; Xia et al., 2004), *B. mori* also became an important bioreactor for production of recombinant proteins (Tomita et al., 2003), vaccines (Li et al., 2008b), enzymes (Li et al., 2011), drugs (Chen et al., 2006). However, a large variety in RNAi efficiency has been reported (Terenius et al., 2011).

Variation in RNAi efficiency exists in Lepidoptera among different species, tissues, stages and target genes (Terenius et al., 2011). This variation might be due to the uptake and delivery of dsRNA, the expression levels of RNAi machinery components and/or presence of RNA degrading enzymes to degrade and clear dsRNA/siRNA/miRNA from the insect body.

Thus, to investigate the variation and efficiency of RNAi response in *B. mori*, several factors were studied in this thesis, including R2D2 which is a siRNA pathway component, dsRNase which is a DNA/RNA non-specific nuclease, and Toll9-1 which is a possible candidate for dsRNA binding. In this thesis, the following subjects were studied:

- Expression pattern of major factors in the siRNA and miRNA pathway in different tissues and at different stages in the silkmoth (Chapter 3)
- Involvement of R2D2 in the RNAi response in the silkmoth Bm5 cells (Chapter 4)
- Functional analysis of dsRNase in dsRNA degradation (Chapter 5)
- Possible interaction between dsRNA and Toll9-1 (Chapter 6 & 7)

Chapter 2

RNAi in *Bombyx mori* (a literature review)

2.1 RNAi in insects

2.1.1 Definition of RNAi

RNAi is a newly identified mechanism of post-transcriptional regulation of gene expression which is highly conserved among high eukaryotes (Carthew and Sontheimer, 2009). It is a process of which exogenous dsRNA silences the complementary endogenous messenger RNA (mRNA) (Zamore, 2001). Since being discovered in the nematode *Caenorhabditis elegans* (Fire et al., 1998), RNAi has rapidly developed as a widely used tool in a variety of insect orders, including Diptera (Dietzl et al., 2007; Lum et al., 2003), Lepidoptera (Chen et al., 2008; Terenius et al., 2011; Tian et al., 2009; Yu et al., 2008), Coleoptera (Arakane et al., 2004; Suzuki et al., 2008) and Hymenoptera (Schluns and Crozier, 2007). Because of its high specificity, RNAi also offers great promise on pest control (Baum et al., 2007; Huvenne and Smagghe, 2010; Price and Gatehouse, 2008).

2.1.2 RNAi pathway

In different organisms, the RNAi pathways comprise different proteins and mechanisms, but they operate by strikingly convergent strategies. In all the organisms that have been studied, RNAi involves two main components: small RNAs that determine the specificity of the response and Agronaute proteins that carry out the repression.

The overall process of RNAi-mediated gene silencing can be divided into three basic steps (Siomi and Siomi, 2009) (Fig. 2.1). First, a long dsRNA expressed or introduced into the cell is processed into small RNA duplexes by a RNase III enzyme known as Dicer. Second, the small RNA duplexes are unwound and one of the strands, known as the guide strand, is preferentially loaded into the RISC containing Argonaute proteins as effectors. Third, the RISC, directed by the guide strand, actively searches target mRNAs containing specific nucleotide sequences complementary to the guide RNA and direct silencing by endonuclease cleavage (siRNAs) or by translation inhibition (miRNAs) (Carthew and Sontheimer, 2009).

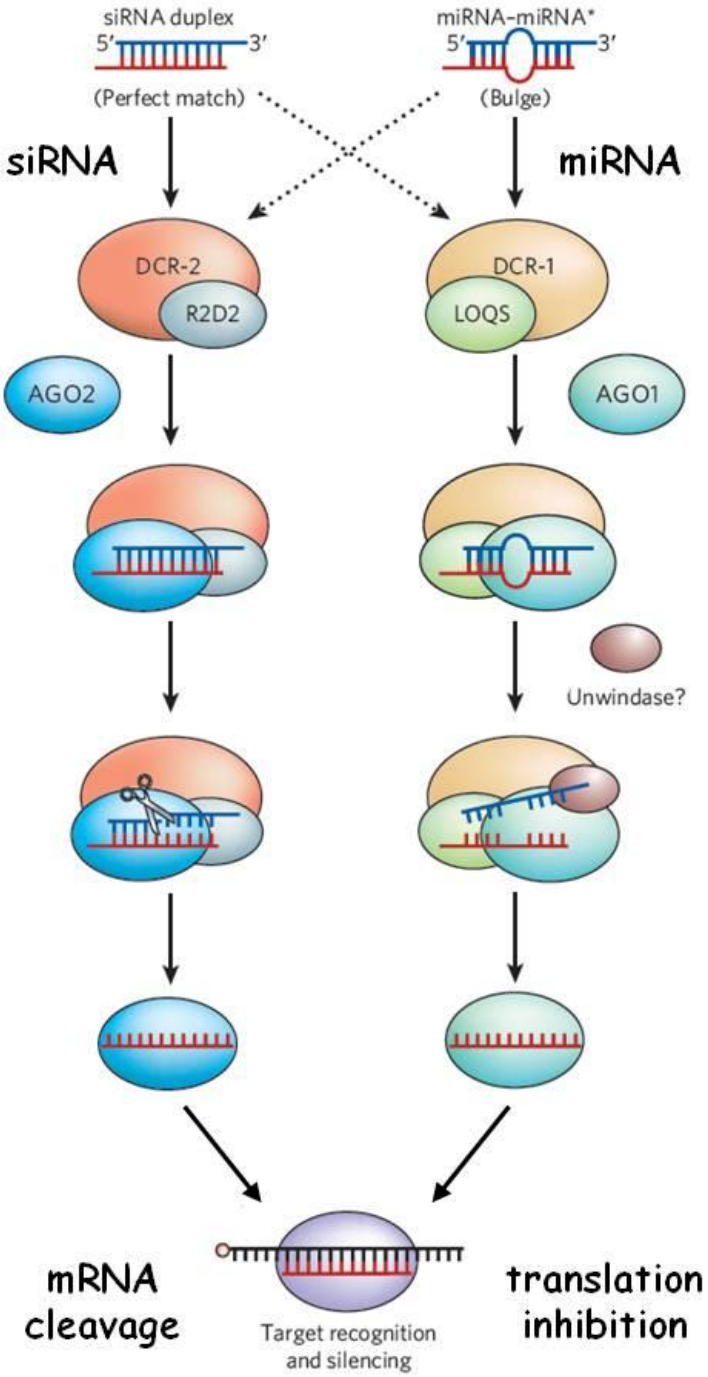


Fig. 2.1. Two main RNAi pathways which include siRNA and miRNA pathways (Siomi and Siomi, 2009).

2.1.2.1 MicroRNAs (miRNAs)

The first miRNA, lin-4 from *Caenorhabditis elegans*, was discoverd in 1993 as an endogenous regulator of genes that controls developmental timing (Lee et al., 1993).

The primary transcripts of miRNAs (primary miRNA, pri-miRNA) are transcribed in the nucleus of the cell by RNA polymorase II or III. These pri-miRNAs are capped and

polyadenylated and contain tandem complementary sequences which allows for the molecule to fold back on itself. This complementary folding generates a partial dsRNA containing one or more miRNA stem-loop structures. The miRNA stems in these pri-miRNAs may contain short single regions of base mismatches (Ding, 2010; Siomi and Siomi, 2009) (Fig. 2.2A).

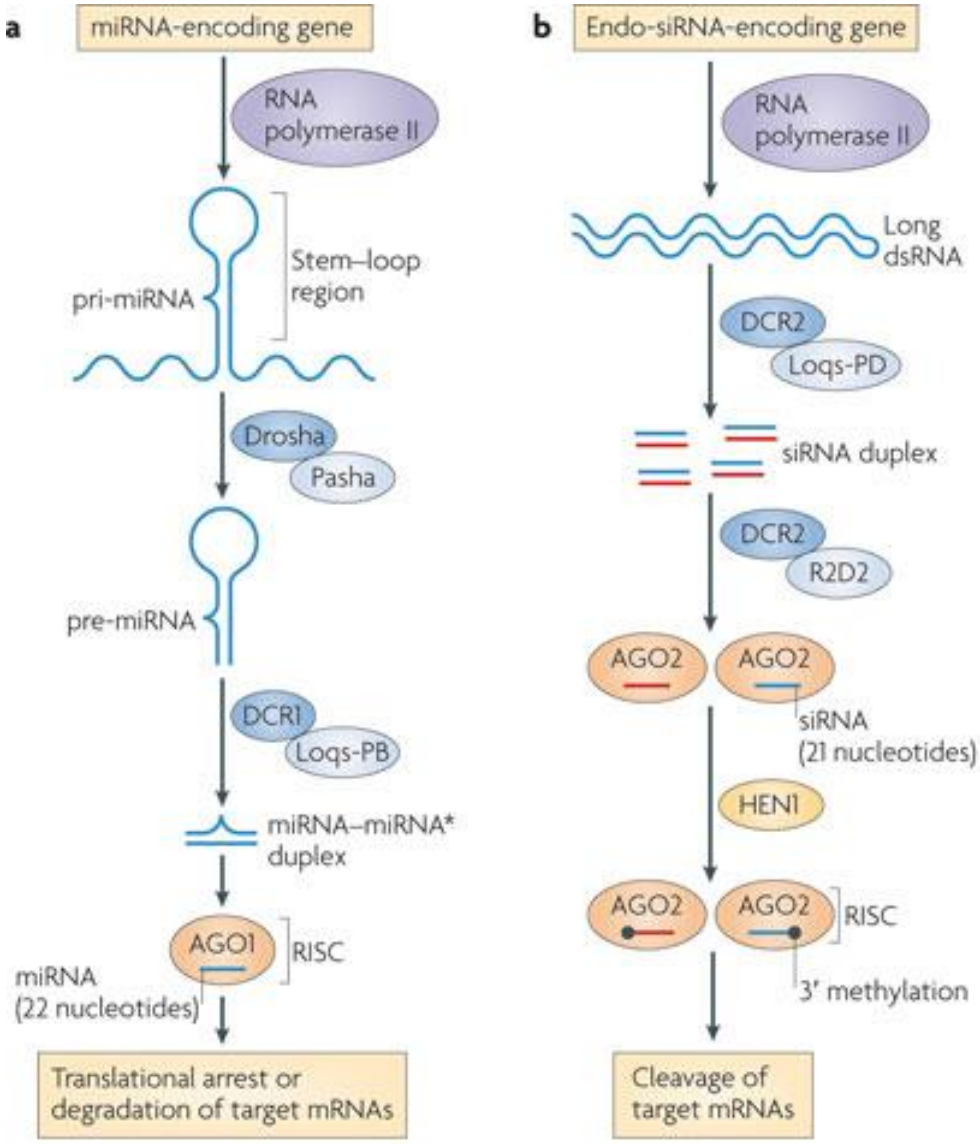


Fig. 2.2. (a) miRNA and (b) siRNA pathways in *Drosophila melanogaster* (Ding, 2010).

- In *Drosophila*, the first processing step occurs in the nucleus. The RNase III enzyme Drosha in combination with the dsRNA-binding protein Pasha forms a complex in the nucleus known as the Microprocessor. The pri-mRNAs are cropped in the Microprocessor to produce a hairpin precursor miRNA (pre-miRNA) of ~65-70 nucleotides in length that has a 2 nucleotide overhang on the 3' end.

- In the second processing step, the stem-looped pre-miRNAs are transported to the cytoplasm by exportin 5 where they are cleaved into short dsRNAs by a second RNase III enzyme known as Dicer-1. In *Drosophila*, Dicer-1 works with an additional dsRNA-binding protein known as Loquacious (Loqs).
- In the third processing step, one of the stands of the dsRNA is referred to as miRNA or guide strand and the other is the miRNA* (miRNA star) or passenger strand. After unwinding, the miRNA strand, which has lower base pairing stability at the 5'-end of the two strands, is loaded onto the miRNA-protein complex RISC by direct binding to the Ago-1 protein. The miRNA loaded RISC (miRISC) is then guided by the miRNA to the specific target mRNAs. The interaction between the miRISC and the target mRNA results in the loss of stability and degradation or inhibition of the translation of the mRNA.

2.1.2.2 Short interfering RNAs (siRNAs)

Fire and Mello reported that exogenous dsRNA could specifically silence genes through a mechanism called RNAi in *C. elegans* (Fire et al., 1998). Very shortly thereafter, the direct conversion of dsRNAs into 21-25 nt siRNAs was documented (Zamore et al., 2000). In insects, the siRNA pathway is regarded to be a principal antiviral pathway and is considered to be part of the insects innate immune system (Merkling and van Rij, 2012). For the *in vivo* RNAi application, most strategies are through introduced exogenous dsRNA into the organisms, thus the siRNA pathway is more practical in RNAi application (Fig. 2.2B).

- **Biogenesis of siRNA**

The generation of siRNA in *Drosophila* is through RNase III enzyme Dicer-2, in which long dsRNA is processed into small ~21-23 bp siRNA duplex. The duplex has a phosphate group at both 5' ends and hydroxyl groups and two nucleotides overhangs at both 3' ends. In this process, Dicer-2 is the key factor that carries out the function.

Prokaryotic RNaseIII carries a single RNaseIII domain and functions as a homodimer (Blaszczyk et al., 2001), whereas eukaryotic Dicer contains two RNaseIII domains and functions as a monomer (Zhang et al., 2004). Through mutagenesis studies of putative catalytic residues of *E. coli* RNaseIII and human Dicer, Zhang et al. (2004) proposed that RNaseIII/Dicer contains a single catalytic center for processing dsRNA. The tandem RNaseIII domains of Dicer form an intramolecular dimer and cleave opposing strands of dsRNA in an offset manner to produce a 2-nt 3' overhang. Similar results were obtained with

corresponding experiments for *Drosophila* Dicer-1 (Ye et al., 2007). In addition, structural and modeling stuides of a primitive Dicer from *Giardia intestinalis* suggested that the PAZ domain recognizes the 3' end of siRNA and the size of siRNA produced is determined by the physical distance between the PAZ and RNaseIII domains (Macrae et al., 2006). Thus, Dicer itself is a molecular ruler.

- **Loading and sorting of siRNA by RISC**

In gene silencing pathways initiated by dsRNA precursors, Dicer-mediated cleavage yields small dsRNA intermediates (small RNA duplexes). These small RNA duplexes must be dissociated into "competent" single strands in order to function as guides for RISC. For each small RNA duplex, only one strand, the guide strand, is loaded onto a specific Argonaute protein and assembled into the active RISC; the other strand, the passenger strand, is destroyed.

For siRNA, the known interactions between Dicer and Argonaute proteins indicate that the production of the small RNA and the assembly of the RISC might be physically coupled (Hutvagner and Simard, 2008). In *D. melanogaster*, Dicer-2 does not simply transfer siRNA to a distinct RISC but, instead, forms part of the RISC together with the siRNA, indicating that the role of Dicer-2 extends beyond the initiation phase. The loading of siRNA duplexes onto Ago2 is facilitated by the RISC-loading complex, which contains Dicer-2 and its dsRBD-containing partner, R2D2 (Liu et al., 2003). R2D2 is thought to sense the thermodynamic stability of the siRNA duplexes and bind to the more stable end of the siRNA, whereas Dicer-2 is recruited to the less stable end. The unwinding of the siRNA duplex and the loading of a single strand into the RISC are facilitated by the slicing of the unincorporated (passenger) strand by Ago2 (Matranga et al., 2005).

Once the passenger strand is cleaved by Ago2 and the guide strand is loaded to the RISC, RISC will start to mediate a range of the effector steps in all RNA silencing mechanisms.

- **Effector modes of the RISC**

RISC was demonstrated as a magnesium-dependent endoribonuclease (Schwarz et al., 2004), of which the main component is Argonaute protein. Argonaute protein (Ago) contains a PAZ doamin, a PIWI domain, middle (Mid) domain and N-terminal (Liu and Paroo, 2010) (Fig. 2.3). The PAZ domain of Ago contains an oligonucleotide-binding fold that interacts

with the 2-nt 3' overhang of siRNA, while the PIWI domain induces the target mRNA degradation. In conjunction with PAZ, PIWI domain, N-terminal and Mid domains of Ago form a crescent base and a positively charged channel, wherein guide siRNA recognizes target mRNA, and the 5' phosphate of siRNA is anchored within the binding pocket of the Mid domain (Tolia and Joshua-Tor, 2007). Consistent with the earlier biochemical studies (Martinez and Tuschl, 2004), structural studies revealed that helical interaction between siRNA and target RNA positioned the scissile phosphate corresponding to the ninth and tenth positions of guide RNA at the catalytic center (Tolia and Joshua-Tor, 2007). Cleavage of target RNA results in a 5' fragment with a 3'-hydroxyl terminus and a 3' fragment with a 5' phosphate (Martinez and Tuschl, 2004).

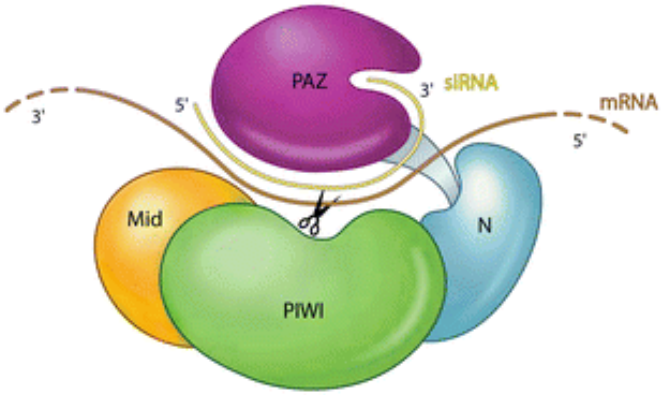


Fig. 2.3. A scheme to represent the domains of Argonaute protein (Ago) and siRNA-directed mRNA cleavage (Liu and Paroo, 2010). The 3' end of siRNA is positioned in the cleft of the PAZ domain. The mRNA situates between the upper PAZ domain and the lower crescent-shaped base formed by the N-terminal, PIWI, and middle (Mid) domains. The catalytic site (scissors) slices mRNA at a position that corresponds to the ninth and tenth nucleotides of guide siRNA.

2.1.3 dsRNA delivery ways

In order to achieve RNAi response, dsRNA should be delivered into the cells of target organisms. Nowadays, different ways of dsRNA delivery methods are applied with successful examples, including direct injection to hemolymph, ingestion by feeding dsRNA or bacteria expressed dsRNA and soaking or transfection in the insect cells.

2.1.3.1 Delivery of dsRNAs through injection

Microinjection was already used by Fire and Mello in the early days of RNAi (Fire et al., 1998) as a way to introduce dsRNA into *C. elegans*. The first microinjection experiments reported in insects were with *D. melanogaster*. Soon after the first publication of RNAi in nematodes, Kennerdell & Carthew (1998) succeeded in downregulating the *frizzled* and

frizzled 2 gene. Then in 2000, the genome of *D. melanogaster* was sequenced and published (Adams et al., 2000), and RNAi became a very popular research tool in functional genomics for this dipteran model insect.

Not long after *D. melanogaster*, a few other insects have also had their genomes sequenced, such as the honeybee *Apis mellifera* (Weinstock et al., 2006) and more recently the red flour beetle *Tribolium castaneum* (Richards et al., 2008). These organisms subsequently became very interesting for RNAi experiments because a lot of gene sequence information became available without any knowledge about their function in question.

Another model organism in the insect class is *T. castaneum*, a stored product pest insect of worldwide importance. Injection of dsRNA, both in larvae and adults, is a widely used technique in functional genomics for this organism, not in the least because, contrary to *D. melanogaster*, *T. castaneum* shows a robust systemic RNAi response (Tomoyasu and Denell, 2004). For *Tribolium* and *Drosophila*, perhaps the two most popular insects as far as these RNAi experiments are concerned, a number of microinjection protocols has been published setting a standard for this kind of work.

Also in Lepidoptera, dsRNA delivery by injection has been proven to be successful, albeit with more difficulties compared to many insects from other classes. An extensive overview of RNAi experiments by microinjection in Lepidoptera has been given by Terenius et al. (2011). Also in *A. mellifera*, economically a hugely important insect and a model organism, belonging to the Hymenoptera, injection of dsRNA has been proven successful to cause a knockdown effect (Aronstein and Saldivar, 2005; Farooqui et al., 2003; Gatehouse et al., 2004).

Besides these model organisms, injection is also used in other species and orders as an important technique for introducing dsRNA into the organism to elicit an RNAi response. In continuation, other successes have been published for the pea aphid *Acyrthosiphon pisum* (Jaubert-Possamai et al., 2007), cockroach *Blattella germanica* (Belles, 2010; Huang and Lee, 2011; Martin et al., 2006), the cricket *Gryllus bimaculatus* (Moriyama et al., 2008; Nakamura et al., 2008) and also in some arachnids such as the tick *Ixodes scapularis* (Narasimhan et al., 2004) and the two-spotted spider mite *Tetranychus urticae* (Grbic et al., 2011; Khila and Grbic, 2007).

The technique has both its advantages and disadvantages compared to the other methods of dsRNA delivery. One important advantage is that it allows researchers to get the dsRNA immediately to the tissue of choice or into the hemolymph and thus avoiding possible barriers such as the integument or the gut epithelium which could be a problem in feeding or

soaking experiments**.** Another advantage is that the exact amount of dsRNA brought into an organism is known, in contrast to delivery by soaking or in some cases by feeding.

However, there are some important disadvantages as well with this method. The work itself is more delicate and time-consuming than the alternatives, and it also requires quite some optimization. Factors like needle choice, optimal volume and place of injection are very important and differ greatly between organisms. For example, in *A. pisum* aphids, the injected volume has proven to be very critical in the survival of aphids after injection (Jaubert-Possamai et al., 2007). Thus, these factors should be carefully optimized before starting any experiment.

2.1.3.2 Delivery of dsRNAs through ingestion

In 1998, Timmon and Fire reported that *C. elegans*, fed on *Escherichia coli* bacteria expressing dsRNA, showed the same phenotypes as the corresponding loss-of-function mutants (Timmons et al., 2001; Timmons and Fire, 1998). This discovery created an extra method to introduce dsRNA into organisms for triggering RNAi. After the initial discovery in this nematode, dsRNA-mediated RNAi research by ingested dsRNA was applied in various insects such as *Spodoptera exigua, Diabrotica virgifera virgifera* and *Epiphyas postvittana* (Baum et al., 2007; Surakasi et al., 2011; Tian et al., 2009; Turner et al., 2006). Two main strategies can be used in dsRNA feeding experiments: dsRNAs can either be expressed in bacteria, or they can be synthesized *in vitro,* and then fed to insects either by mixing with food or by supplying as solution droplets.

Besides its application in *C. elegans*, bacterially expressed dsRNAs were also used in research on insects. The *E. coli* HT115(DE3), expressing dsRNAs corresponding to *S. exigua* chitin synthase A (SeCHSA) gene, was overlaid on the pieces of artificial diet pellets which were fed to *S. exigua* larvae and thus dsRNAs were introduced in the gut (Tian et al., 2009). The bacterial dsRNA-fed larvae showed a disturbed development and higher mortality compared with the control as well as and a reduced SeCHSA gene expression. Notably, the SeCHSA gene is a non-midgut gene, specifically expressed in the cuticle and tracheae of *S. exigua*. The success of gene silencing through ingested dsRNA suggested that systemic RNAi could be induced, which is consistent with the existence of a SID-1 like gene in *S. exigua.* Uptake of dsRNA by feeding also induced transcriptional suppression in salivary glands of the tick *I. scapularis* (Soares et al., 2005) and fat body tissue of *Reticulitermes flavipes* (Zhou et al., 2008b). Furthermore, three different doses of bacterial culture were tested for RNAi efficiency in *S. exigua*. As expected, higher mortality and the highest reduction in mRNA

expression were observed in larvae fed on a high dose of dsSeCHSA, while lower mortality and lack of knockdown of the SeCHSA transcript were found in low dose dsRNA-fed larvae, suggesting that sufficient knockdown of target gene requires a sufficient dose of dsRNA. Moreover, reduction of SeCHSA transcript was detected on the 7th day after ingestion of dsRNA, rather than on the 3rd or 5th day, suggesting a requirement of accumulation of dsRNA molecules for triggering RNAi.

dsRNAs can also be synthesized *in vitro* and the dsRNA solution can be easily mixed with food for oral delivery to insects. dsRNAs of a species-specific E-subunit of the vATPase gene of *T. castaneum*, *A. pisum* and *M. sexta* were synthesized in cell-free condition by Whyard et al. (2009). dsRNA solutions were dissolved in liquid artificial food or overlaid on the surface of solid foods for these insects. The ingestion of vATPase dsRNA led to 50-75% mortality for all three insects.

In contrast, neither artificial diet that was surface coated with dsRNA nor food that was mixed with dsRNA could induce RNAi in four *Drosophila* species (*D. melanogaster, D. pseudoobscura, D. sechellia, D. yakuba*) over a 5-days period of larval development (Whyard et al., 2009). Interestingly, similar levels of RNAi as the above three insects (*T. castaneum*, *A. pisum* and *M. sexta*) were observed of the *Drosophila* larvae by dsRNA droplet-feeding as well as transfection reagents-mediated dsRNA solution-soaking (Whyard et al., 2009). In the study on β1 integrin subunit (*βSe*1) in *S. exigua*, cabbage pieces soaked in dsRNA solution were fed as food to 4th-instar larvae of *S. exigua*, which transiently suppressed the expression of *βSe*1 in the digestive gut epithelium and this led to significant mortality (Surakasi et al., 2011).

Another way of oral dsRNA delivery is the dsRNA droplet-feeding as described by Turner et al. (2006). Here 48 h-starved 3rd-instar larvae of *E. postvittana* were fed on dsRNAs derived from the larval gut carboxylesterase gene (*Epos*CXE1) and the adult antennae-expressed pheromone binding protein (*Epos*PBP1) gene. In particular, the silencing of *Epos*PBP1 indicated the existence of systemic RNAi in *E. postvittana* and persistence of dsRNA across the life stage from larva to adult, which agreed with previous studies by Misquitta & Paterson (1999) and Amdam et al. (2003). This droplet-feeding method was also applied in the research on a cytochrome P450 (CYP6BG1) gene in *Plutella xylostella*, demonstrating successful knockdown of the target gene and significant RNAi effects on the corresponding phenotype (Bautista et al., 2009). Capillary tube feeding of dsRNA solution in *I. scapularis* ticks provides another ingestion way of introducing dsRNA with effective RNAi effect being observed (Soares et al., 2005).

More recently, a novel way of delivering dsRNA in a large scale was reported as nanoparticle-mediated RNAi. dsRNAs were entrapped by the polymer chitosan via electrostatic forces to form a chitosan/dsRNA nanoparticle (Sarathi et al., 2008; Zhang et al., 2010). These nanoparticles can be delivered into the insect by ingestion. The innovation of this method is the formation of nanoparticles that stabilize the dsRNA molecules through the delivery process and thus enhancing the efficacy of RNAi.

Recent studies demonstrated that transgenic plants can be engineered to express hairpin dsRNAs targeting genes from insects to increase their resistance to herbivorous insects (Baum et al., 2007; Mao et al., 2011). Delivery of dsRNAs through transgenic plants has been studied to effectively silence genes in insects of Lepidoptera, Coleoptera and Hemiptera (Baum et al., 2007; Pitino et al., 2011; Zha et al., 2011). The plants selected in these studies were transgenic models such as rice *Oryza sativa* (Zha et al., 2011), tobacco *Nicotiana tabacum* (Mao et al., 2007), *Arabidopsis thaliana*, and cotton *Gossypium hirsutum* (Mao et al., 2011). Transgenic cotton plants (*G. hirsutum*) expressing dsCYP6AE14 acquired enhanced resistance to cotton bollworm, which indicated the usefulness of RNAi technology in engineering an insect-proof cotton cultivar (Mao et al., 2011). However, the suitable genes for transgenic plants should not only be biologically important to the targeted insects but also safe to other non-targeted animals and humans. Fortunately, large scale gene screening by RNAi through ingestion could offer a convenient way to achieve these goals (Tian et al., 2009).

Oral delivery of dsRNA into insects provides several advantages. It is labor-saving, cost-effective and easy to perform (Tian et al., 2009). Also, this method is extremely applicable for high throughput gene screening, especially genes for pest control (Kamath et al., 2001). Another advantage of oral delivery of dsRNA is that it is less invasive and also a more practical method for small insects such as aphids and 1^{st}- and 2^{nd}-instar larvae or nymphs (Tian et al., 2009). However, delivery of dsRNA by ingestion has several limitations as well. As observed in previous research, ingestion of dsRNA is less effective for inducing RNAi in *C. elegans* (Hunter, 1999) and *Rhodnius prolixus* (Araujo et al., 2006) than injection. Furthermore, ingested dsRNAs targeting a gut-specific aminopeptidase N failed to induce RNAi in *Spodoptera litura* (Rajagopal et al., 2002) which suggests that the oral delivery of dsRNA may not be suitable for all species, as discussed before. Moreover, the efficiency of RNAi by ingestion of dsRNA varies between different species possibly due to a different gut environment. Therefore, optimization of the used concentration of dsRNA to trigger RNAi is important (Turner et al., 2006). Another limitation of oral delivery of dsRNA

is that it is hard to determine the amount of dsRNA brought inside the insect through ingestion (Surakasi et al., 2011), which could compromise many investigations.

2.1.3.3 Delivery of dsRNAs through soaking and transfection

In recent years, soaking the organism into a dsRNA solution, using the extracellular RNAi, seems to be a popular method for triggering RNAi response as well, mainly because of its convenient operation. The first experiment regarding soaking was reported by Tabara *et al*. (1998) in which they found that specific RNAi was induced by simply soaking of nematodes (*C. elegans*) in the dsRNA solution. Thus, this technique was applied for large scale analysis of gene function in this species to accomplish high throughput RNAi (Maeda et al., 2001).

In insects, most of the soaking experiments were reported for experiments with insect cell lines. The first cell line used for soaking experiment was the S2 cells derived from *D. melanogaster* embryos (Clemens et al., 2000). By direct addition of specific dsRNA to the cell growth medium, suppression of specific gene expression was reported (Caplen et al., 2000). Subsequently, soaking became the most commonly used method to induce RNAi response in S2 cells (March and Bentley, 2007; Shah and Forstemann, 2008). Since then, this technique has become widely used for RNAi experiments in many other insect cell lines. In Sf21 cells derived from ovaries of *Spodoptera frugiperda* (Valdes et al., 2003), downregulation of target genes was accomplished by soaking the cells in dsRNA (Sivakumar et al., 2007) and siRNA (Agrawal et al., 2004) solutions.

However, Beck and Strand (2005) found that by simply adding dsRNA to the culture medium of Hi5 cells (derived from *Trichoplusia ni*), no RNAi response was observed. In contrast, introduction of the dsRNA into the cells by transfection did cause a downregulation of the target genes. Transfection-mediated gene silencing using intracellular RNAi was also reported in Sf21 cells (Valdes et al., 2003). It seems that transfection causes a more efficient RNAi response compared to simply soaking, most likely because the introduction into the cell is more efficient.

Recently, a cell line from *Chrysodeixis includens* embryos (CiE1) was established for dsRNA uptake. Transfection of dsRNA in CiE1 cells was reported to suppress the expression of target genes (Johnson et al., 2010). Although the Sl2 cell line (derived from *Spodoptera littoralis*) was also used for dsRNA uptake, no efficient silencing was observed in these lepidopteran cells as was also the case for the Bm5 cell line (derived from *B. mori*) (Terenius et al., 2011).

Other factors that help to trigger RNAi effects in cell lines were also reported. In contrast to *C. elegans*, which internalize dsRNA by the channel-forming transmembrane protein SID-1 (Winston et al., 2002), the mechanism of dsRNA uptake into *D. melanogaster* cells is through endocytosis mediated by Scavenger receptors such as SR-CI and Eater. These two scavenger receptors accounted for more than 90% of the dsRNA uptake into S2 cells (Ulvila et al., 2006). However, overexpression of the SID-1 protein of *C. elegans* in *Drosophila* S2 cells could help the transport of dsRNA into the cells (Feinberg and Hunter, 2003).

Although the RNAi effects are less potent than those obtained by direct microinjection, soaking appears to work with the similar efficiency compared to feeding in *C. elegans* (Tabara et al., 1998). This method seems to be more convenient and easily handled. Therefore, it allows to perform high throughput RNAi screens to problems in cell and developmental biology (Perrimon and Mathey-Prevot, 2007), and it could be applied to genome-wide analysis in the study of phenotypes characterization (Sugimoto, 2004). This large-scale RNAi analysis has dramatically increased the amount of gene functional information (Perrimon and Mathey-Prevot, 2007). However, delivery of dsRNA by soaking is more applicable for insect cells than for whole insect bodies, probably due to certain barriers such as the insect cuticle.

Despite the extra barriers, uptake of the dsRNA by whole insect bodies is possible. Direct spray of dsRNA on newly hatched *Ostrinia furnalalis* larvae was performed by Wang *et al.* (2011), which resulted in considerable mortalities ranging from 40% to even 100% after treatment and this effect correlated with the down-regulation of the target gene expression as verified by qPCR. This easy and applicable delivery method demonstrated the possibility of dsRNAs penetrating the insect integument and triggering RNAi. RNAi-based pest control can be facilitated by this high-throughput dsRNA delivery method.

2.1.4 RNAi in *B. mori*

The first RNAi in *B. mori* was published in 2002, which reported the knockdown of a pigment gene following dsRNA injection into embryos (Quan et al., 2002). Since then, micro-injection of dsRNA into embryos has most often been used for *B. mori*, and in all cases successfully (Liu et al., 2008; Masumoto et al., 2009; Pan et al., 2009; Quan et al., 2002; Suzuki et al., 2012; Tomita and Kikuchi, 2009). Micro-injection seems to be a popular dsRNA delivery way in *B. mori*. So far, most of the successful examples seem to be related to developmental processes and immunity, including embryonic development (Liu et al., 2008;

Masumoto et al., 2009; Mrinal and Nagaraju, 2008; Quan et al., 2002; Suzuki et al., 2012), postembryonic development (Dai et al., 2008; Gui et al., 2006; Hossain et al., 2008; Huang et al., 2007), embryonic and larval coloration (Quan et al., 2002), cocoon pigmentation (Tabunoki et al., 2004), immune system (Gandhe et al., 2007) and sex pheromone synthesis (Hull et al., 2009; Hull et al., 2010; Ohnishi et al., 2009; Ohnishi et al., 2006).

Other successful RNAi experiments were reported in the two studies of transgenic *B. mori* expressing hairpin RNAs, where high levels of silencing of the target gene were described (Dai et al., 2008; Kanginakudru et al., 2007). Electroporation-mediated somatic transgenesis was also reported by using short hairpin RNA (shRNA)-mediating DNA vectors and direct transfer of siRNAs, in which RNAi effect was also achieved (Ando and Fujiwara, 2012).

Nevertheless, the RNAi efficiency in *B. mori* is not as robust as in *Tribolium* (Tomoyasu et al., 2008). There were some failures in the RNAi experiments, e.g., injection of dsRNA targeting the ecdysone receptor or other target genes in the epidermis did not result in silencing effects (Terenius et al., 2011).

As for the *Bombyx* derived Bm5 cells, silencing of the homologous gene was not achieved by direct adding dsRNA to the culture medium (Terenius et al., 2011). However, the channel transport protein SID-1 from *C. elegans* could mediate the uptake of dsRNA into *Bombyx* BmN4 cells (Kobayashi et al., 2012; Mon et al., 2012).

In the dsRNA delivery ways, it seems that ingestion of dsRNA has not been tested in *B. mori*, while micro-injection and soaking of cells were tested with some successful and unsuccessful examples. This variation might be due to the uptake of dsRNA, which was proved to be a factor since SID-1 could facilitate this process and achieve RNAi effect (Kobayashi et al., 2012; Mon et al., 2012), the expression of RNAi machinery core genes (Tomoyasu et al., 2008), or the presence of dsRNA-degrading enzymes (Terenius et al., 2011).

2.1.5 RNA-degrading enzymes

In all organisms tested from all kingdoms of life, RNA degradation is a prevalent activity. It has become clear that cells transcribe more RNA than they accumulate, implying the exitence of active RNA degradation systems (Houseley and Tollervey, 2009). There are three major classes of intracellular RNA degrading enzymes (ribonucleases or RNases) existing in the organisms. Endonucleases that cut RNA internally, 5'-exonucleases that

hydrolyze RNA from the 5'-end, and 3'-exonucleases that degrade RNAfrom the 3'-end (Houseley and Tollervey, 2009).

In the RNAi process, these RNA-degrading enzymes could affect the RNAi efficiency in nematodes and insects. Both positive and negative effects have been reported on RNAi efficiency in different organisms.

- The *eri-1* gene, which was first characterized in *C. elegans*, encodes a 3' to 5' exonuclease of the DEDDh superfamily of RNase T exonucleases (Kennedy et al., 2004). Mutations of *eri-1* in *C. elegans* cause an enhanced RNAi (ERI) phenotype (Kennedy et al., 2004), which means that *eri-1* is a negative regulator of RNAi. On the other hand, its involvement in the accumulation of endogenous siRNAs was also reported (Lee et al., 2006) and also in the fission yeast *Schizosaccharomyces pombe*, loss of the ERI-1 homolog resulted in increased levels of siRNAs (Iida et al., 2006).
- Tudor staphylococcal nuclease (Tudor-SN) contains five staphylococcal nuclease-like (SN) domains and a Tudor domain and it was proven to be the first RISC subunit which possessed a recognizable nuclease domain (Caudy et al., 2003). Functional analysis showed it was involved in cleavage of dsRNA and it is also a ribonuclease specific for degradation of miRNA precursors (Li et al., 2008a). Knockdown of Tudor-SN in nematode (Caudy et al., 2003) and shrimp (Phetrungnapha et al., 2011) resulted in an impairment of the RNAi effect, indicating the requirement of Tudor-SN for proper function of the RISC.
- Another positive RNAi regulator that was recently identified is a protein complex called C3PO (component 3 promoter of RISC) which was shown to be a Mg^{2+} dependent endoRNase (Liu et al., 2009) . In *Drosophila*, C3PO, composed of two conserved proteins: Translin and Trax (Ye et al., 2011), has been shown to promote the RISC activity by facilitating endonucleolytic cleavage of the siRNA passenger strand (Liu et al., 2009; Tian et al., 2011).
- Double-stranded ribonuclease (dsRNase), a DNA/RNA non-specific alkaline nuclease, is a dsRNA-processing enzyme. However, the major characteristic of this class of enzymes is their broad substrate specificity. Homologs show highest activity towards dsRNA but are also able to digest ssRNA, ssDNA, dsDNA, as well as RNA/DNA hybrids (Meiss et al., 1999; Siwecka, 1997). Homolog studies also indicated its involvement in mitochondrial DNA replication (Cote and Ruiz-Carrillo, 1993), DNA repair (Dake et al., 1988) and nucleic acid conformation (Nestle and Roberts, 1969; Robertson et al., 1968). However, its role in RNAi process remains to be clarified.

2.1.6 Immune response besides RNAi

RNAi is a cellular process to silence specific genes, in which exogenous dsRNA is recognized and the complementary endogenous mRNA is degraded. This mechanism is also an evolutionary conserved defense response against virus infection or dsRNA structure molecules, but virus or dsRNA could also trigger other pathways in the immune response besides RNAi (Merkling and van Rij, 2012) (Fig. 2.4).

Insects first appeared on earth approximately 350-400 million years ago and they are composed of more than 70% of the animal species today (Mayhew, 2007). One of the reasons for this successful evolution is the effective innate immune system in the insects. Unlike the vertebrate animals that have innate and acquired immune systems, insects only have the innate immune system which has been shown to respond to bacteria, fungi, parasites, viruses etc. (Evans et al., 2006).

There are several signal transduction pathways in insects induced by pathogens (Merkling and van Rij, 2012), which includes Toll pathway, immune deficiency (IMD) pathway, Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway and c-Jun N-terminal kinase (JNK) pathway (Merkling and van Rij, 2012; Tanaka et al., 2008). These inducible immune pathways allow insects to rapidly respond to the invading pathogens which are defined as pathogen-associated molecular patterns (PAMPs), such as bacteria, fungi and viruses, after recognition by receptors on the cellular membranes. This recognition immediately activates respective immune signal pathways and triggers the transcription of downstream genes afterwards, which leads to the release of the effector molecules, especially the antimicrobial peptides (AMPs) which become highly expressed in the fat body and hemocytes (Table 2.1) (Christophides et al., 2002; Ferrandon et al., 2007; Lemaitre, 2004; Lemaitre and Hoffmann, 2007; Reichhart et al., 1993; Tauszig et al., 2000).

The fruit fly is a powerful model to study innate immunity. Over the years, detailed insight in antibacterial and antifungal immunity has been obtained, greatly facilitated by the extensive genetic toolkit for this model organism (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). The above immune transduction pathways have been extensively studied in this organism.

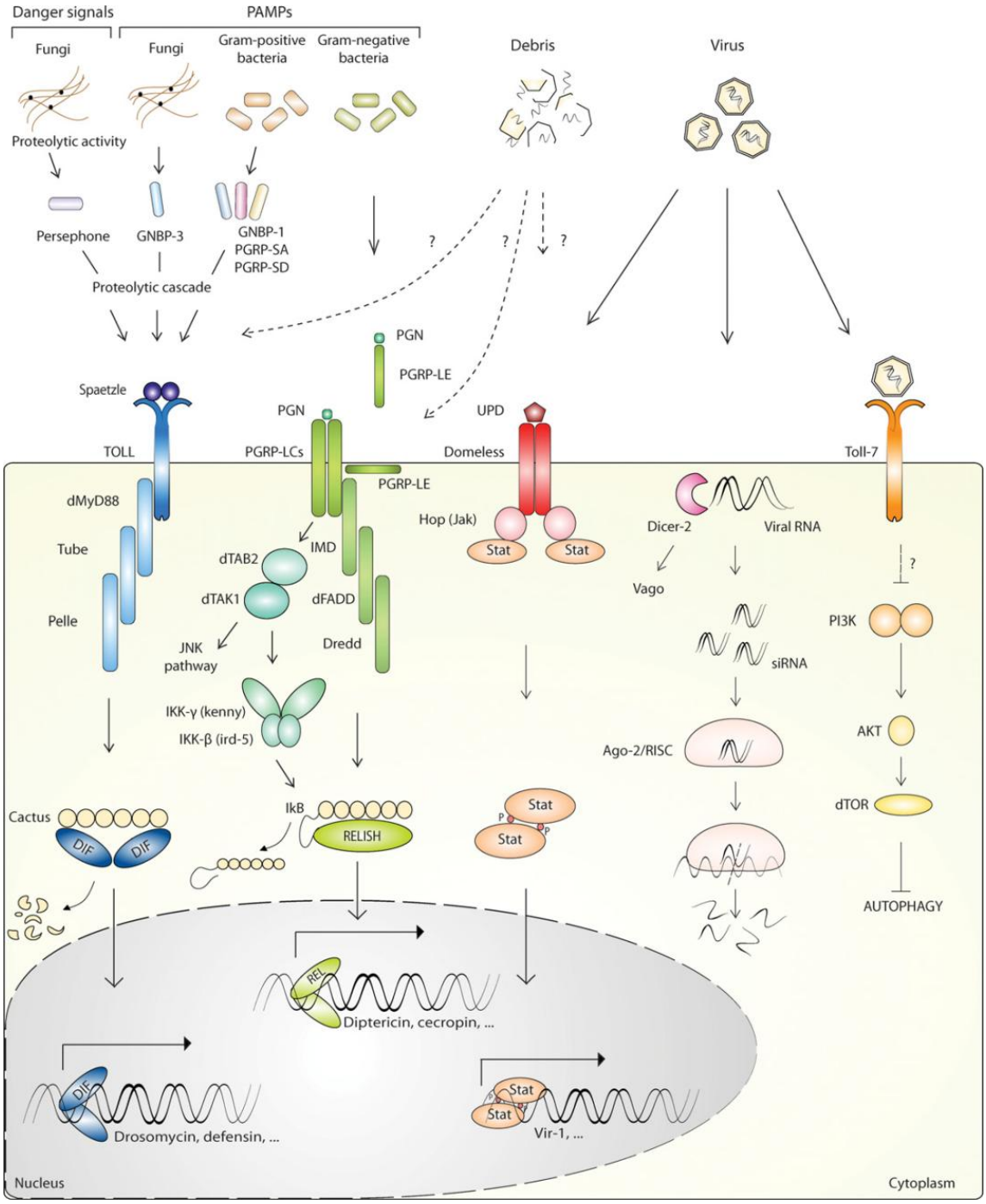


Fig. 2.4. Inducible immune pathways in *Drosophila* (Merkling and van Rij, 2012). Toll pathway: detection of pathogen-associated molecular patterns (PAMPs) of fungi (beta-glucans) and Gram-positive bacteria (Lys-type peptidoglycan) or danger signals (such as proteolytic activity in the hemolymph) triggers a proteolytic cascade, leading to cleavage of the precursor of the dimeric cytokine Spätzle. Mature Spätzle binds to the membrane-anchored Toll receptor, thereby inducing its dimerization. Three intracellular death domain-containing proteins, MyD88, tube, and pelle are then recruited. The kinase pelle is likely responsible for phosphorylation of cactus, thereby directing its degradation by the proteasome. Dif (Dorsal-related immunity factor) is then able to translocate to the nucleus, where it induces transcription of immune genes, including the antimicrobial peptides (AMPs) *Drosomycin* and *Defensin*. Imd pathway: Binding of microbial diaminopimelic (DAP)-type peptidoglycan (PGN) from Gram-negative bacteria to peptidoglycan recognition proteins (PGRP-LC or PGRP-

LE) induces the recruitment of the adaptor molecules Imd (Immune deficiency) and *dFADD* (*Drosophila* Fas-associated death domain). This leads to activation of the caspase DREDD (Death related ced-3/Nedd2-like protein) and of TAK1 (Transforming growth factor-β-activated kinase 1). TAK1 and its adaptor TAB2 (TAK1-binding protein 2) activate a complex of IκB Kinase (IKK)-*β* and IKK-*γ* (also known as *ird-5* and *kenny*, respectively), which then directs phosphorylation of Relish, followed by its proteolytic cleavage by DREDD. The inhibitory domain (IκB) of Relish remains stable in the cytoplasm, whereas the *Rel* domain of Relish translocates to the nucleus and induces transcription of immune genes, such as the AMPs *Diptericin* and *Attacin*. PGRP-LE can act as an intracellular receptor, and, in a truncated form, as an extra-cellular receptor. Jak-Stat pathway: upon virus infection, the Janus kinase (Jak) - signal transducers and activators of transcription (Stat) pathway is activated, presumably by binding of an unpaired (Upd) cytokine to the dimeric domeless receptor. The Jak tyrosine kinase Hopscotch (Hop), in association with domeless, then phosphorylates both itself and the cytoplasmic tail of domeless, creating binding sites for Stat92E. Upon binding, Stat92E is phosphorylated, dimerizes and translocates into the nucleus where it induces transcription of target genes that contain stat binding sites in their promoters, such as *virus induced RNA-1* (*vir-1*). RNAi: viral double-stranded RNA (dsRNA) is recognized and processed by Dicer-2 into small interfering (si) RNAs, which are then incorporated in an Argonaute-2 (Ago-2) containing RNA-induced silencing complex (RISC). Within RISC, these siRNAs guide the recognition and cleavage of complementary viral RNA sequences, thereby restricting virus replication. Autophagy: vesicular stomatitis virus binds to the transmembrane receptor Toll-7 and induces autophagy. This is likely mediated through negative regulation of the phosphatidylinositol 3-kinase (PI3K)-Akt kinase pathway. It is likely that cell debris or damaged virus-infected cells release or act as immunostimulatory damage-associated molecular patterns that activate immune signaling pathways via undefined mechanisms.

Table 2.1. AMPs from *Bombyx mori*. Names, types belonging to, gene numbers, and main activity against Gram-positive bacteria or Gram-negative bacteria are shown (Tanaka and Yamakawa, 2011).

Name	Type	Gene number	Main activity
Attacin	Glycine-rich	2	Gram-negative
Cecropin	Amphiphilic-alpha-helix	13	Gram-positive, Gram-negative
Defensin	Six cysteine containing	2	?
Lebocin	Proline-rich, O-glycosylated	1	Gram-negative
Moricin	Amphiphilic-alpha-helix	1	Gram-positive, Gram-negative
Gloverin	Glycine-rich	4	Gram-negative
Lysozyme		1	Gram-positive

In *D. melanogaster*, the Toll signaling pathway plays an important role in immunity and development against Gram-positive bacteria and fungi (Aggarwal and Silverman, 2008; Merkling and van Rij, 2012). In *D. melanogaster*, the Toll pathway is activated by Spätzle, the only ligand for the Toll receptors that has been identified so far (LeMosy et al., 1999). Spätzle is present in *Drosophila* (six genes), *Anopheles* (six genes), *Apis* (two genes),

Bombyx (three genes) and *Manduca* (one gene) (An et al., 2010; Tanaka et al., 2008). Spätzle binds to the extracellular domain of the Toll receptor (Arnot et al., 2010). The activated Toll receptor binds to the adaptor protein Myeloid differentiation factor 88 (MyD88) via intracellular Toll/IL-1 receptor (TIR) domains (Horng and Medzhitov, 2001). Upon this interaction, Tube and Pelle are recruited to form a complex through their death domain (Moncrieffe et al., 2008), which in turn leads to the phosphorylation of the IκB factor, Cactus, a negative regulator of NF-κB signaling (Wu and Anderson, 1998). After phosphorylation-induced degradation of Cactus, the translocation of the NF-κB transcription factors, Dorsal and Dif, from cytoplasm into the nucleus leads to activation of AMP genes (Reichhart et al., 1993).

On the contrary, the IMD signaling pathway is mainly initiated by a diaminopimelic type peptidoglycan (DAP-type PGN) from Gram-negative bacteria, which is recognized by the transmembrane protein, peptidoglycan recognition protein LC (PGRP-LC) (Choe et al., 2002). Upon this recognition, the adaptor proteins IMD (Georgel et al., 2001) and *Drosophhila* FAS-associated death domain-containing protein (*d*FADD) are recruited (Zhou et al., 2005). This recognition leads to the activation of the caspase, Death related ced-3/NEDD2-like protein (DREDD), which in turn directs the phosphorylation of Relish, followed by the proteolytic cleavage by DREDD (Silverman et al., 2000). The inhibitory domain (IκB) of Relish remains in the cytoplasm, while the Rel domain of Relish is translocated to the nucleus, resulting in the induction of AMP genes (Aggarwal and Silverman, 2008).

The JAK/STAT signaling pathway is involved in antibacterial and antiviral immunity (Merkling and van Rij, 2012). This pathway is activated via the binding of the extracellular protein ligand Unpaired (Upd) to the receptor Domeless (Dome) (Goto et al., 2010). Upon binding, the JAK kinase, Hopscotch (Hop), is recruited which leads to the phosphorylation of the STAT transcription factor, STAT92E (Agaisse et al., 2003; Yan et al., 1996). Phosphorylated STAT92E is then translocated to the nucleus to direct the transcription of immune genes with STAT-binding sites in their promoter (Agaisse and Perrimon, 2004).

Besides the above-mentioned signaling transduction pathways, RNAi is also regarded in the context of the antiviral immune response, which causes gene specific silencing (Karpala et al., 2005; Kemp and Imler, 2009). In this process, dsRNAs or viral genomes with dsRNA structure are cleaved by Dicer enzymes into siRNAs. Subsequently, these siRNAs are loaded into RISCs that scan RNAs for complementary sequences and degrade mRNA and viral RNA (Carthew and Sontheimer, 2009; Siomi and Siomi, 2009). Although the antiviral

defense mechanisms of insects remain poorly understood, many studies on antiviral immunity have been performed in *Drosophila* and lots of virus have been identified nowadays.

Accumulation of Flock house virus (FHV) siRNAs in infected *D. melanogaster* cells provided the first experimental evidence for the induction of RNA silencing that targets virus infection in an animal host (Li et al., 2002). These FHV siRNAs are distinct from the subsequently discovered virus-derived mRNAs in invertebrates and vertebrates, which are processed from specific sites and polarity of the viral genome (Pfeffer et al., 2004). The FHV-derived siRNAs are likely to have an antiviral role during infection of *D. melanogaster* because clearance of FHV genomic RNA and subgenomic RNA from infected cells requires Ago2, which has been shon to load siRNAs derived from synthetic dsRNA into the RISC (Fig. 2.5). Subgenomic RNA refers to the RNA transcripts of the viral RNA genome that contain only part of the sequence present in the entire genome and usually funcion as mRNA. Consistent with this finding, the FHV B2 protein, which is a viral suppressor of RNA silencing (VSR), is essential for FHV infection but becomes dispensable after depletion of Ago2 in *D. melanogaster* cells (Li et al., 2002).

Subsequent studies have detected virus-derived small RNAs and RNA-based antiviral immunity in fungi, plants, *D. melanogaster*, mosquitoes, silkmoths and *C. elegans*. the target viruses have many different types of genome, including (+)RNA, negative-strand RNA ((-)RNA), dsRNA, single-stranded DNA (ssDNA) and dsDNA.

Analysis of the role of the siRNA pathway pathway was greatly facilitated by the fact that flies mutant for Dicer-2, Ago2, or R2D2 are viable (Liu et al., 2003). These mutant flies succumb more rapidly than wild-type controls to infection with RNA viruses *Drosophila* C virus (DCV), Cricket paralysis virus (CrPV), FHV, or Sindbis virus (SINV). Increased lethality correlates with increased viral titers in infected flies. Because DCV and CrPV (*Dicistroviridae*), FHV (*Nodaviridae*), and SINV (*Alphaviridae*) belong to different families of viruses, these data indicate that the siRNA pathway mediates a broad antiviral defense in flies (Kemp and Imler, 2009). Intrestingly, resistance to the virus *Drosophila* X virus (DXV; *Birnaviridae*) and West Nile virus (WNV; *Flaviviridae*) seems to involve AGO2, but not Dicer-2 (Chotkowski et al., 2008; Zambon et al., 2006). In addition, PIWI-interacting RNA (piRNA) pathway, a related but distinct small RNA silencing pathway, has also been implicated in antiviral defense recently (Merkling and van Rij, 2012). piRNA pathway appears to mediate resistance to these two viruses, since mutants for Piwi, Aubergine, or the helicase Spindle E are more sensitive to DXV and/or WNV. Hence, antiviral RNAi may involve more than one pathway.

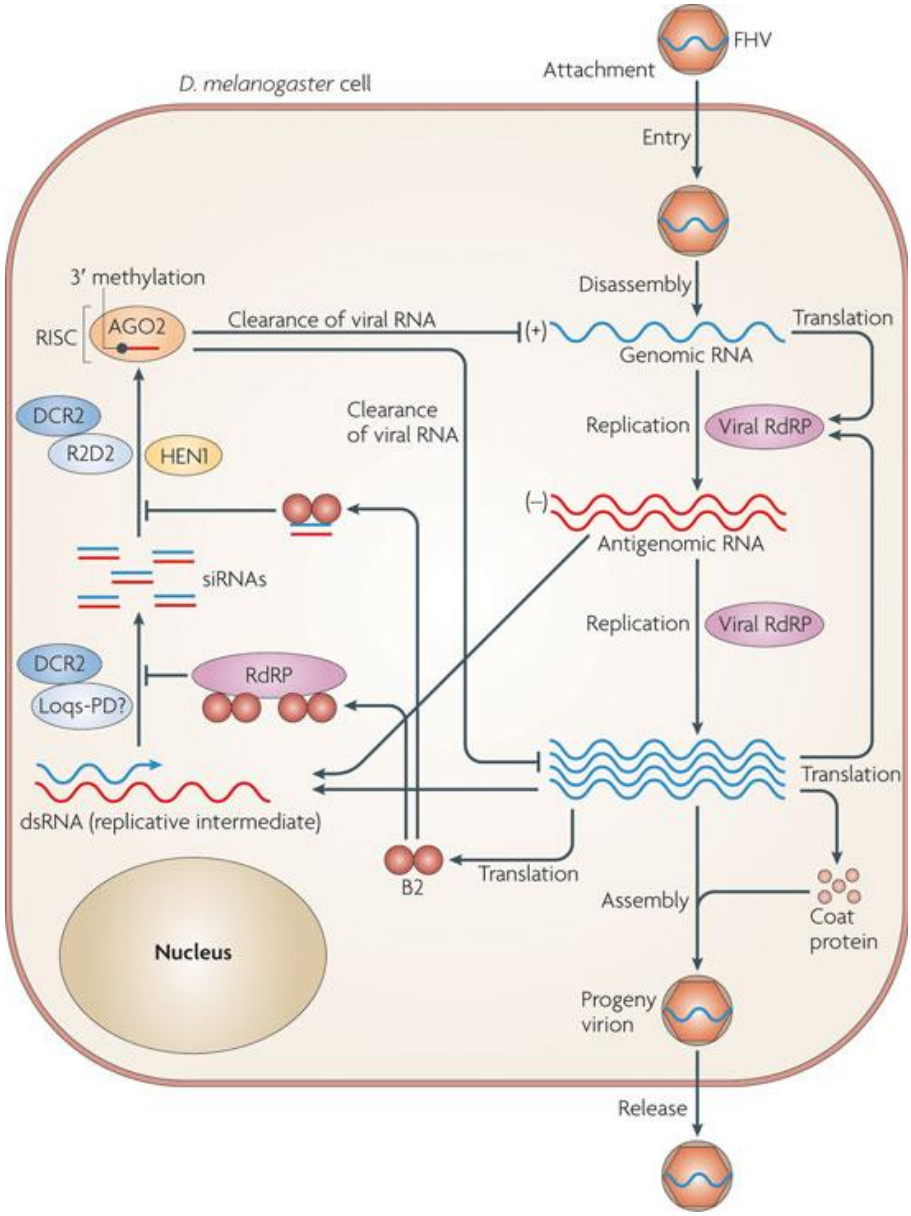


Fig. 2.5. Key steps in RNA-based antiviral immunity induced in *D. melanogaster* by infection of positive-strand RNA viruses such as flock house virus (Ding, 2010). Following entry and uncoating of flock house virus (FHV) virions, the genomic positive-strand RNA ((+)RNA) serves as both mRNA for the translation of viral RNA-dependent RNA polymerase (RdRP) and as a template for the synthesis of antigenomic negative-strand RNA ((−)RNA). Preferential production of (+)RNA by viral RdRP is achieved by multiple rounds of initiation of RNA synthesis from the 3' end of the low abundant (−)RNA. The resulting double-stranded RNA (dsRNA) formed between the 5'-terminal nascent progeny (+)RNA and the (−)RNA template is recognized by Dicer 2 (DCR2) and cleaved into small interfering RNAs (siRNAs), thereby triggering RNA-based antiviral immunity. The viral siRNAs are assembled with Argonaute 2 (AGO2) into the RNA-induced silencing complex (RISC), methylated at the 3' end (depicted by a black circle) by HEN1 and used to guide specific clearance of FHV RNAs. As a counter-defence, FHV encodes a viral suppressor of RNA silencing (VSR), the B2 protein, which targets two steps in this immune pathway: inhibition of viral siRNA production by binding to viral RdRP and the viral dsRNA precursor, and sequestration of viral siRNAs by binding duplex siRNAs. Loqs-PD, loquacious-isoform PD.

2.2 The domesticated silkmoth*Bombyx mori*

2.2.1 Taxonomy of *B. mori*

Superkingdom	Eukaryota
Kingdom	Metazoa
Phylum	Arthropoda
Superclass	Hexapoda
Class	Insecta
Subclass	Neoptera
Infraclass	Endopterygota
Order	Lepidoptera
Suborder	Glossata
Superfamily	Bombycoidea
Family	Bombycidae
Subfamily	Bombycinae
Genus	*Bombyx*
Species	*Bombyx mori*

2.2.2 General introduction

The silkmoth (*Bombyx mori* L.) (Fig. 2.6) is a domesticated insect, which feeds mainly on mulberry (*Morus alba*) leaves to produce raw silk in the form of cocoon. It is an economically important insect, being a primary producer of silk. It is entirely dependent on humans for its reproduction and does not occur naturally in the wild. Sericulture, the practice of breeding silkmoths for the production of raw silk, has been underway for at least 5000 years in China, from where it has spread to Korea and Japan, and later to India and the West.

Fig. 2.6. The silkmoth larva (Daizo strain) at the 5th instar.

2.2.3 Physical description

The larvae of *B. mori* are caterpillars that are about 4 cm long, including their horned tail. They are buff-colored with brown thoracic markings. The adults are moths with a 4 cm wingspan. They are also buff-colored, but have thin brown lines on their whole bodies (Herbison-Evans, 1997).

2.2.4 Life cycle

The life cycle of silkmoths has four distinct stages of development, which completes one generation of the species, including egg, larva, pupa and moth (Fig. 2.7).

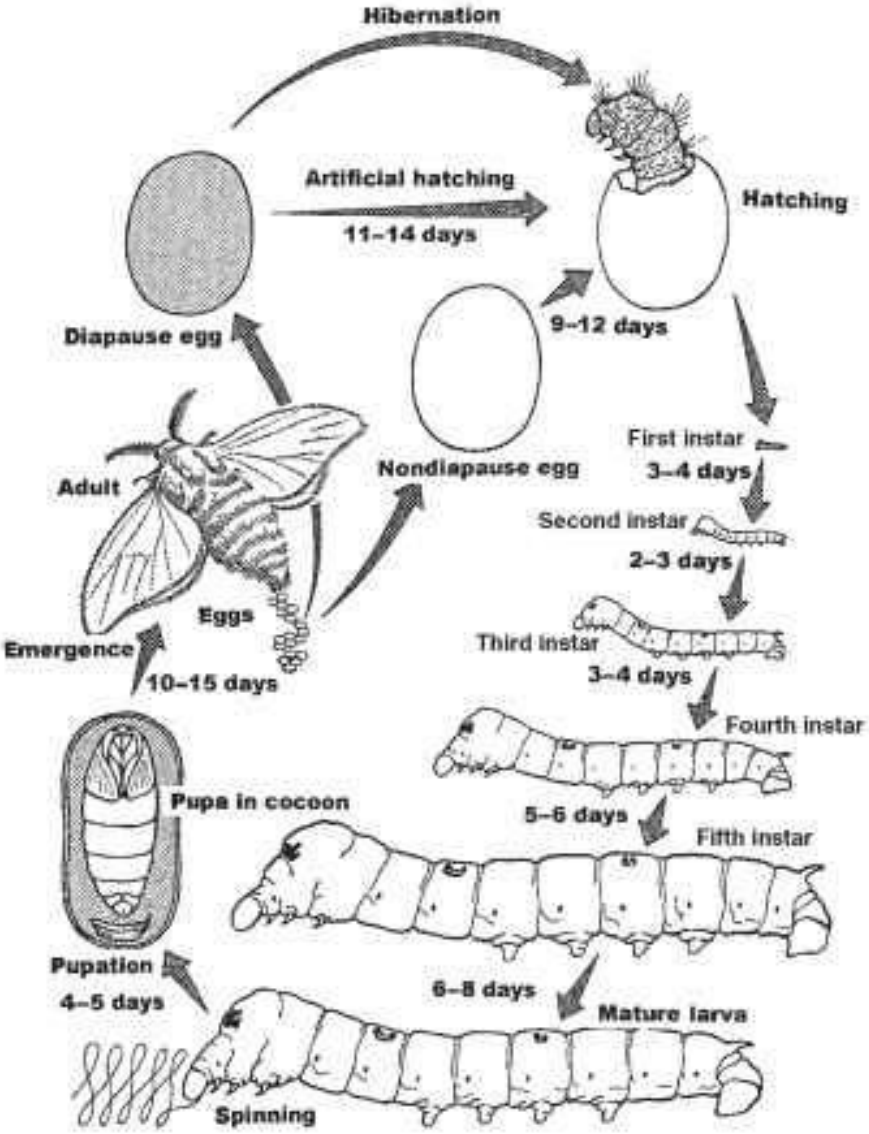


Fig. 2.7. Life cycles of the silkmoth, adapted from Mori (1970).

Domesticated mulberry silkmoths are univoltine, bivoltine and multivoltine. Univoltine silkmoth completes one generation, bivoltine completes two generations and multivoltine silkmoth completes 5-6 generations per year. The univoltines lay diapausing eggs and only hatch once in a year at spring season. Bivoltines lay both type of eggs, diapausing eggs and non-diapausing eggs which hatch in spring season and autumn season. Multivoltines do not lay diapausing eggs (Table 2.2).

Table 2.2. The life stages of mulberry silkmoths

Stages	**Sub-stages**	**Duration (days)**
Eggs	Multivoltine or non-diapausing	10-11
	Univoltine or diapausing	Over wintering
Larvae	I Instar	3-4
	II Instar	2-3
	III Instar	3-4
	IV Instar	5-6
	V Instar	6-8
Pupae	Cocooning	2-3
	Pupation	2-3
	Emergence	4-7
Adults	Life	3-4

Eggs:

The size, weight, shape and color of the eggs vary with the silkmoth strains, season and nutrition of mother moths at larval stage. Generally, the eggs are ovoid, spherical or ellipsoid in shape and flat on one side. The female moth lays 400-500 eggs on mulberry leaves or artificially prepared paper. 100 eggs weigh about 60 mg. The silkmoth eggs hibernated during winter will hatch in spring when the mulberry sprouts. Generally, the non-diapausing eggs hatch in 10-11 days at proper temperature and humidity condition.

Larvae:

The larvae are polypodous or eruciform type, which could be divided into three parts, including the head, thorax and abdomen (Fig. 2.8). The head is hypognathous, heavily chitinized and bright brown in color. There is a spinneret, as the median process, at the distal

portion of the labial prementum through which silk glands open and silk exudes. It has three pairs of ocelli or simple eyes. The thorax bears 3 pairs of conical short, jointed true legs and the prothorax has a pair of spiracles laterally. The abdomen has nine visible segments as the 9th,10th and 11th segments are fused to form the apparent ninth segment that bears a paired anal prolegs. The 3rd, 4th, 5th and 6th abdominal segment bears paired abdominal prolegs. The dorsum of the 8th segment has a projection called caudal horn. The first eight abdominal segments have a pair of spiracles laterally.

The larvae have five instars which go through four moults. The newly hatched larvae are black in color and covered with bristles and thus resemble tiny black ants at a glance so called ant worms. The larvae in the first instar are about 3 mm long with 13 segments.

The larvae become smoother and lighter during different succeeding instars. Among five larval instars, the first three are known as young larvae and the last two instars are called grown-up larvae. The larvae raise their head and search a shelter for resting and secrete silken thread and cover themselves with cocoon.

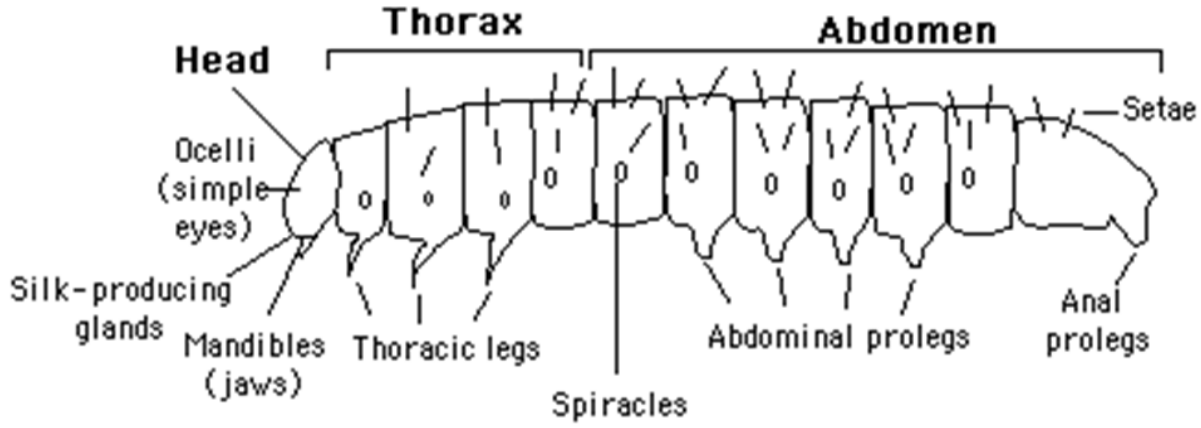


Fig. 2.8. The anatomy scheme of a silkmoth larva (http://www.enchantedlearning.com/subjects/butterfly/activities/printouts/silkworm.shtml).

Pupae:

Pupae are soft and white soon after the moult which occurs within the cocoon spun by the final instar larva. It takes 2-3 days to spin the cocoon and then it transforms into pupae in 2-3 days and after about 1 week it emerges as a moth. The pupa is a non-motile and non-feeding stage. The color of the pupal cuticle, compound eyes, and wing pads change with the age of the cocoon and is useful for the gross examination of the age of the pupae

Adults:

From the cocoon the moth emerges and emergence takes place in the morning. Copulation also takes place just after emergence. The moths have prominent bipectinate antennae and compound eyes on the head. The coiled proboscis is non functional. There are three pairs of thoracic legs and two pairs of wings in the thorax region. Moths cannot fly but males are more active than females in movement. If adults copulate in captivity, the female will lay eggs within 24 hours.

2.2.5 Food habits

A silkmoth is a herbivore and its preferred food is mulberry leaves, but it may also eat the leaves of any other mulberry tree (i.e., *M. rubra* or *M. nigra*) as well as the Osage orange (*Maclura pomifera*). The quality and quantity of mulberry leaves play important roles in the growth and development of silkmoth, particularly during the larval stage, which in turn influence the expression of cocoon productivity traits. This also leads to the increase in the size of body and dry weight of cellular mass which are dependent on the rate of metabolism, absorption of nutrients, and stage of development. The larval stage of silkmoth is the most important stage in the food requirement, mulberry leaves consumption takes place during the larval stage of silkmoth. So, considerations should be made in fulfilling the required amount of food (mulberry leaves) at the proper stage of time.

Because the silkmoths have been cultivated for so long for sericulture (the silk industry), they have lost an adaptation helpful to feeding in the wild. The larvae can no longer hang on plants at gravity-defying angles, and must be fed by humans. In the sericulture industry, artificial diet is also used to feed the larvae.

2.2.6 Artificial diet

Mulberry leaves are the natural diet for silkmoths and the leaves must be given freshly enough to meet the preference of larvae, therefore it has to be given to the silkworm 3 or 4 times a day. Such rearing system raises some problems: 1) high cost in field. Because mulberry field, rearing houses and farmer's habitation must be located together at somewhere transportation is convenient. 2) Intensive labor. Since labor requirement of mulberry harvesting and silkworms rearing peak at the same time. 3) Seasoning limitation. In the winter, mulberry trees do not grow leaves to supply the silkmoth larvae, thus rearing the larvae in the winter is impossible. These demerits might limit further sericulture

development. A system of rearing silkworm with artificial diet may solve the above problems.

The first artificial diet for insect was developed by Bogdanow from Russia in 1908 to reared a blowfly, *Calliphora vomitoria*, on an artificial diet of meat extract, starch, peptone and mineral salts (Bogdanow, 1908). In 1960, Fukuda *et al.* firstly succeeded to rear the silkmoth from egg to moth on an artificial diet (Table 2.3). The dietary compositions have been improved continually since then. Nowadays, hundreds of the diets for various purposes have been developed. Rearing silkmoth larvae on artificial diets become more and more popular in sericulture.

The application of artificial diet in sericulture has many advantages such as: 1) The silkworm can be reared throughout the year; 2) labor can be saved in mulberry leaves harvesting; 3) disease infection can be reduced from the field.

Table 2.3. Composition of the artificial diet developed by Fukuda in 1960

Substances	Content (g)
Mulberry leaf powder	5.0
Starch	1.5
Sucrose	1.0
Quina powder	1.0
Defatted Dofu	1.0
Antiseptics	Added
Distilled water	15ml

Generally, the artificial diets can be divided into following three basic types:

- **Oligidic diets**

This type of diet is consisted principally of crude natural materials such as mulberry leaf powder. The diets are usually used in mass rearing projects when the components are readily accessible and inexpensive. Oligidic diets are popular because of the general simplicity in preparation and their low cost. Some compositions of this type of diest are given on Table 2.4.

Table 2.4. Compositions of some oligidic diets for silkmoth larvae (g)

Substances	Ito (1968)	Miao (2000)	Zhang (1992)	Chen (1994)
Mulberry leaf powder	50	30	30	35
Defatted soybean meal	20	30	35	35
Potato starch	20	-	-	-
Corn starch	-	20	11.5	11
Soybean residue powder	-	-	15	-
Sucrose	-	4	-	-
Cellulose	8	6	-	10
Ascorbic acid	2	-	1.5	1.5
Citric acid	0.5	3	2	2
Sitosterol	-	-	0.4	0.4
Salt mixture	-	2	3	3
Vitamin B mixture	Added	1	1.0	1.5
Crotonic acid	-	-	0.4	0.4
Agar	12	3	-	-
K_2HPO_4	1	-	-	-
Antiseptic	Added	0.05	Added	added

- **Meridic diets**

This type of diets is also called semi-synthetic diets. The components are composed mostly of defined chemicals, but with one or more undefined components, such as defatted soybean meal. Diets showed in Table 2.5 may be regarded as examples of the meridic diets.

Table 2.5. Compositions of meridic diets (g) (Ito,1974)

Substance	(1)	(2)	(3)
Potato starch	10.0	10.0	20.0
Sucrose	10.0	10.0	-
Glucose	-	-	12.0
Soybean meal,defatted	30.0	40.0	60.0
Soybean oil, refined	3.0	3.0	3.0
Sitosterol	0.5	0.5	0.5
Salt mixture	3.5	3.5	2.0
K_2HPO_4	1.0	1.0	-
Ascorbic acid	2.0	2.0	2.0
Cellulose powder	34.0	34.0	-

Agar	15.0	15.0	5.0
Citric acid	0.5	0.5	0.5
Sorbic acid	0.2	0.2	0.2
Morin	0.2	0.1	-
Total	109.9	119.8	105.2
Vitamin B mixture	Added	Added	Added
Antiseptic	Added	Added	Added
Dist water	300ml	300ml	260ml

Note: (1) 1st instar; (2) 2nd-4th instar; (3) 5th instar.

- **Holidic diets**

This type of diets is consisted entirely of known pure chemicals, but by the strictest definition, a holidic diet for silkmoth larvae may not exist. Contaminants are commonly present in many of the components (such as agar) used in the diet. Perhaps the amino acid diet (Table 2.6) may be regarded as one of the holidic diets. The holidic diets are most desirable for critical nutritional studies.

Table 2.6. Composition of amino acid diet (Ito, 1966)

Substance	**Quantity (g)**	**Substance**	**Quantity (g)**
Potato starch	5.0	Mulberry leaf fraction	10mg
Sucrose	15.0	Biotin	4μg
Amino acid mixture	20.0	Choline chloride	1500μg
Soybean oil, refined	3.0	Folic acid	4μg
Sitosterol	0.5	Inositol	400μg
Wesson's salt mixture	4.0	Nicotinic acid	300μg
Ascorbic acid	2.0	Ca-pantothenate	150μg
Cellulose powder	35.0	Pyridoxine-HCl	30μg
Morin	0.3	Riboflavin	20μg
Agar	15.0	Thiamine-HZ	20μg
Total	100.0	Sorbic acid	added

An available artificial diet should possess the following four conditions:

(1) The components can meet the nutritional requirements for the silkmoth larvae.

(2) The physical properties are suitable for the feeding and acting of the larvae.

(3) The prepared diet must be stable and antimicrobial. Thus antiseptics should be

added to the diets in preparation.

(4) The proper feeding inducers should be added. Supplying the silkworms with a nutritionally adequate diet is not enough for the fastidious insect that does not feed on a diet without appropriate phago-stimulants. The behavior-modifying chemical stimulants can be divided into 3 groups: 1) Attractants including citral, linalyl acetate, linalol, terpinyl acetate etc. (Hamamura, 1975); 2) Bite-inducers including morin, isoquercitrin, β-sitosterol; 3) Gorging stimulants: cellulose, sugar, inositol, vitamin C, chlorogenic acid, phosphate, silicate etc.. Suitable regulation of the dietary acidity to pH 4~5 with citric acid and ascorbic acid also favors the feeding activity of the larvae. Mulberry leaf powder in the oligidic diets provides the larvae with most of the necessary feeding inducers. But some more chemicals such as gallic acid, inositol, citric acid and as well as vitamin C, phosphate, cellulose, sugar and β-sitosterol are added in some diets.

2.2.7 Silkmoth genome and public databases

Silkmoth has 28 chromosomes and is estimated a haploid nuclear genome size of 530 Mb. Recently, a large and diverse collection of genetic markers, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphic (RFLP) and simple sequence repeat (SSR), have been developed and mapped. The combined map contains more than a thousand molecular markers, at an average spacing of 2 cM (or about 500 Kb). In addition, among the more than 400 visible phenotypes, nearly 200 are assigned to linkage groups. Interestingly, some genes related to cocoon color, virus resistance and wingless mutation have been positionally cloned and analyzed.

The silkmoth genome project experienced three phases. Firstly, a large number of expressed sequence tag (EST) sequences for silkmoth multiple tissues have been sequenced and released in 2003 (Mita et al., 2003). Secondly, 6x and 3x draft genome sequences were independently generated based on whole genome shotgun (WGS) sequencing by Chinese and Japanese groups in 2004, respectively (Mita et al., 2004; Xia et al., 2004). Finally, Chinese and Japanese groups worked jointly and used the merged WGS reads, together with newly generated fosmid- and BAC-end data, to assemble a high-quality genome sequence of the silkmoth in 2008 (Consortium, 2008; Yamamoto et al., 2008). The new silkmoth genome assembly has a size of 432Mb and has the best continuity (~ 15.5Kb and 3.7Mb in N50 contig and scaffold size, respectively) (Consortium, 2008). Using a high-density single nucleotide polymorphism (SNP) linkage map consisting of 1,577 markers, about 87.4% of the scaffold sequences were anchored to all 28 chromosomes (Yamamoto et al., 2008).

Among the 767 cDNAs collected from GenBank, 96% (738) could be fully aligned on the genome with correct order and exon orientation. The estimated gene count is 14,623, which is slightly higher than *Drosophila melanogaster* (14,039 genes) and 44% higher than *Apis mellifera* (10,157 genes). Moreover, approximately 47% of silkmoth genes have EST expression evidence, 38% have GO classifications, and 76% have identifiable *D. melanogaster* homologues.

To access the silkmoth genome sequence data, there are three databases which are available and widely used nowadays, inclusing SilkDB (http://www.silkdb.org/silkdb/), SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi) and KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/).

To manage the silkmoth genome sequence data, the SilkDB (V1.0) was constructed by Chinese group in 2004 after the completion of draft sequence (Wang et al., 2005; Xia et al., 2004). Along with the performation of silkmoth functional genomics research, huge functional data of the silkmoth genes have produced. To better deposit the newly assembled silkmoth genome sequence and related functional data and to facilitate users to access the relative information, a new version of SilkDB (V2.0) was redeveloped (Duan et al., 2009). At the same year after the completion of another independent draft sequence (Mita et al., 2004), the Japanese group also developed a silkmoth databse SilkBase. However, the above databases are not sufficient for building long genomic scaffolds which are essential for unambiguous annotation of the genome. Therefore, the two groups decided to merge and assemble the two datasets through a joint collaboration (Shimomura et al., 2009). This comprehensive database provides effective data mining and efficient utilization of the silkmoth genome information for functional and applied genomis. The genomic sequences, map information and EST data are compiled into an integragted silkmoth genome database KAIKObase, which consists of 4 map viewers, a gene viewer, sequence search, and keyword and position search systems to display results and data at the level of nucleotide sequence, gene, scaffold and chromosome.

2.2.8 Economical and biological importance

The silkmoth has been used for silk production for about 5,000 years. Currently，it is one of the most economically important, beneficial insects in many developing countries owing to its ease of large-scale propagation and textile-industry utilization.

The silkmoth is a pioneering animal in the history of biology, leading to the first verification of the Mendel's Law in animals (1906), the first discovery of molting hormone (1940), and the first identification of pheromone (1959). Also, it is unique in being a domesticated insect that is completely dependent on humans for survival and reproduction. Yet, it could hybridize with its nearest wild relative, making it an excellent model for studying the genetics of domestication. With the development of biotechnology, *B. mori* has become an important bioreactor for production of recombinant proteins (Tomita et al., 2003), vaccines (Li et al., 2008b), enzymes (Li et al., 2011), drugs (Chen et al., 2006) et al.. On the other hand, silkmoth is also one of the best-characterized models for insect biochemical, genetic and genomic studies, because of its large size, easy rearing in the laboratory, complex metabolism and the abundance of mutants. Especially after the publication of the whole genome sequence in 2004 (Mita et al., 2004; Xia et al., 2004), which makes it an ideal insect for genetic and molecular research.

Chapter 3

Expression patterns of key factors involved in different RNAi pathways*

* Adapted from:

Luc Swevers, Jisheng Liu, Hanneke Huvenne, Guy Smagghe (2011). Search for limiting factors in the RNAi pathway in silkmoth tissues and the Bm5 cell line: the RNA-binding proteins R2D2 and Translin. PLoS One 6: e20250.

3.1 Introduction

In *Drosophila*, the two RNA silencing pathways, that involve siRNAs and miRNAs, respectively, seem to be separated with respect to their biogenesis and function. Both types of small RNAs are generated by separate Dicer enzymes (nuclear Drosha and cytoplasmic Dicer-1 for miRNAs and Dicer-2 for siRNAs) and enter RISC assembly pathways with separate Argonaute proteins as core constituents (Ago-1 for miRNAs and Ago-2 for siRNAs) (Tomari et al., 2007). The Dicer enzymes generally also function as a complex with dsRNA-binding proteins that are specific to each pathway (Drosha/Pasha and Dicer-1/Loquacious for miRNA pathway and Dicer-2/R2D2 for the siRNA pathway) (Carthew and Sontheimer, 2009; Tomari et al., 2007). In general, it is thought that the miRNA pathway primarily uses endogenous products from the cell's genome with dsRNA structure (transcribed from endogenous genes as stem-loop precursors) to regulate developmental processes, while the siRNA pathway is a defence response against exogenous dsRNAs, as for instance generated from viruses. More recently, it was recognized that endogenous long inverted repeat transcripts with full complementarity enter a "hybrid" RNA silencing pathway that is dependent on Dicer-2 and Ago-2 as expected, but also involves the Dicer-1-cofactor Loquacious (Okamura et al., 2008). This finding illustrates that siRNA and miRNA pathways can be partially overlapping in *Drosophila*.

Besides the above mentioned *in vitro* systems derived from *Drosophila*, nothing is known regarding the functioning of the RNAi machinery in other insects, including *Tribolium* which is known for its sensitivity to RNAi. In the case for lepidopteran insects, such knowledge could be valuable, as it could lead to the development of new methods to increase RNAi efficiency in this order. To gain insight in the mechanism of the RNAi process in lepidopteran insects, we have carried out gene expression studies of the major factors of the siRNA and miRNA pathways in different tissues and at different stages in the silkmoth. In addition, we have focused on the silkmoth-derived Bm5 cell line which is derived from ovarian tissue (Grace, 1967). This cell line can be easily transfected and transformed (Swevers et al., 2004) and therefore genetically manipulated to test functions of individual factors in small RNA signalling pathways.

3.2 Materials and methods

3.2.1 Experimental animals and cell line

The larvae of *B. mori*, Daizo strain, were reared on artificial diet (Yakuruto Co., Japan) at 25°C under a photoperiod of 12 h light and 12 h dark. Larval tissues were dissected

from 5th instar larvae at day 4-5 after the molt. Tissues from 3-10 larvae were collected in Eppendorf tubes on ice and samples were frozen at -70ºC until further processing for RNA extraction. To isolate hemocytes, hemolymph was collected on ice after cutting the first proleg and subsequently centrifuged at low speed (800 g) at 4ºC to collect the cell pellet. The following tissues were used in the subsequent experiments: epidermis (EP), fat body (FB), midgut (G), thoracic muscles (M), Malpighian tubules (MT), brain (Br), silk glands (SG), testis (T), ovaries (O) and hemocytes (H).

Pupal tissues from 3-5 pupae were collected from pupae at day 5-7 after pupation: wing disk (WD), fat body (FB), midgut (G), Malpighian tubules (MT), brain (BR), testis (T), ovaries (OV).

Bm5 cells were maintained in IPL-41 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and grown at 27°C.

3.2.2 Identification of the RNAi machinery genes

The core machinery genes of the miRNA pathway: Drosha, Pasha, Dicer-1, Loquacious and Ago-1 (Siomi and Siomi, 2009), and the siRNA pathway: Dicer-2, R2D2 and Ago-2 (Kemp and Imler, 2009) were selected to study the expression patterns. Of these genes, Loquacious, Ago-1, Dicer-2, R2D2 and Ago-2 have been annotated in the *Bombyx* genome: respective accession numbers are NM_001195079, AB332314, NM_001193614, NM_001195078 and NP_001036995. To identify homologs of Drosha, Pasha and Dicer-1, tBLASTn searches were carried out using amino-acid sequences from *Drosophila*, *Tribolium* or *Apis mellifera* as query. In all cases, a single sequence with high sequence identity/similarity (e-value of 10^{-18} or lower) was identified which was used to design primers to detect homologous sequences in *Bombyx*.

3.2.3 RNA extraction and reverse-transcription PCR (RT-PCR)

Total RNA from at least three larval tissues was extracted using the TRI Reagent (Sigma, MO) according to the manufacturer's protocol. The quantity of extracted RNA was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, DE) and/or by electrophoresis on 1% (w/v) agarose gels. First-strand complementary DNA (cDNA) synthesis was performed using a SuperScript II reverse transcriptase (Invitrogen, CA). An overview of all primers used to detect the mRNAs of the above core factors in the small RNA silencing pathways is presented in Table 3.1.

All the genes were optimized with different cycle numbers and gradient temperature. PCR was carried out at 35 cycles of amplification, while BR2D2 was amplified for 40 cycles. Control PCRs employed primers to amplify *Actin* mRNA for 30 cycles (Machado et al., 2007). PCR products were run on 1.5% agarose gels and stained with ethidium bromide.

3.2.4 Sequencing of the PCR fragments of the RNAi machinery genes

Amplified PCR fragments were ligated to pJET1.2 cloning vector (Fermentas) in a ratio of 3:1 (insert/vector). After ligation, the mixture was transformed to *Escherichia coli* competent cells by heat shock method (42°C for 90 s) and plated in the LB agar plates overnight at 37°C. Recombinant colonies were picked up from the plates and analyzed by colony PCR: 95°C 3 min; 94°C 30 s, 60°C 30 s, 72°C 1 min/kb; 25 cycles. Colonies with correct inserts were selected for plasmids purification using E.Z.N.A. Cycle Pure Kit (Omega). One microgram of purified plasmids was sent for sequencing (LGC Genomics).

Table 3.1. Primer pairs used for amplification of factors of the core machinery of small RNA pathways

Gene	Forward primer	Reverse primer
miRNA pathway		
BmDrosha	CGTTCACGGATCGCTCAGTC	GCGGAAACAGACACGCGTTG
BmPasha	GTCAGCACCAGCTGGAAGAC	ACTGCATGCCGCCGATGTAC
BmDicer-1	TGAAGCCGGGTGAGGTGTTC	GGGAGTGAGGAGGTTGAGTC
BmLoquacious	GAGCTGTTGGCACGTCGTGG	GTTGTCCTGTAAGGCCTGCC
BmAgo-1	GGGCGATAGCATGTTTCGCG	TCACGTCCACGCCCAGGAAG
siRNA pathway		
BmDicer-2	CATACAGTTCACCGAAGAGG	GGATGTACGACGAGTGAGAC
BmR2D2 (1)	CAAGATGAAAACTCCCATAACAGTACTG	TTTGTCGCGCCTGTCGCTTG
BmR2D2 (2)	CAAGATGAAAACTCCCATAACAGTACTG	TCACAGAGCGGCGGGCGGCGGA
BmR2D2 (3)	AAAGCGCCCACAGTGGACAG	TCACAGAGCGGCGGGCGGCGGA
BmR2D2 (4)	CCGCTGTAAGGCTTTAGGTGAG	TCACAGAGCGGCGGGCGGCGGA
BmR2D2 (5)	AAAGCGCCCACAGTGGACAG	TTTGTCGCGCCTGTCGCTTG
BmAgo-2	TCTCCGATTGACTTGGGCGAC	ATACGGTCATCCTAACCGGCG

3.3 Results

3.3.1 Expression studies of key factors in miRNAs pathways

As is evident from Fig. 3.1, almost all the factors involved in the miRNAs silencing pathways have a very broad expression pattern of their mRNAs. Only the mRNA expression of the *Bombyx* Pasha homolog showed clear differences among different tissues, while differences in expression of other factors are generally much less obvious.

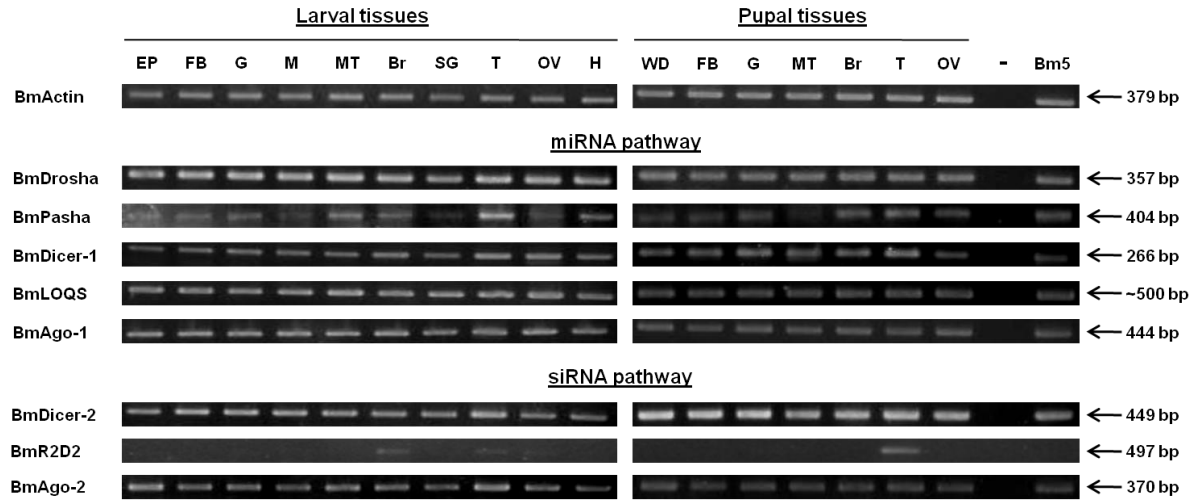


Fig. 3.1. *In vivo* expression pattern study of core machinery genes in the small RNA pathways from different larval and pupal tissues from the silkmoth strain Daizo and the silkmoth-derived Bm5 cell line. The products were obtained after 35 cycles of PCR, with the exceptions of *Actin* which was amplified after 30 cycles, and *BmR2D2* which was amplified for 40 cycles. Abbreviations: larval tissues: EP = epidermis; FB = fat body; G = midgut; M = thoracic muscles; MT = Malpighian tubules; Br = brain; SG = silk glands; T = testis; OV = ovaries; H = haemocytes; pupal tissues: WD = wing disk; FB = fat body; G = midgut; MT = Malpighian tubules; Br = brain; T = testis; OV = ovaries. The column "-" shows amplifications in the absence of template with the exception of the reaction with BmR2D2 primers that shows the fragment amplified from genomic DNA.

3.3.2 Expression studies of key factors in siRNAs pathways

As shown in Fig. 3.1, all the factors involved in the siRNAs pathways also have a broad expression pattern except *Bombyx* R2D2 (BmR2D2). To our interest, the expression of BmR2D2 is very low to absent, despite the presence of the gene in the *Bombyx* genome and the annotation of its mRNA in Genbank (accession number NM_001195078).

3.3.3 Detection of BmR2D2 mRNA

The detection of BmR2D2 mRNA was attempted using different sets of primers (Table 3.1). Using primer pair 1, PCR products of different sizes were detected. In one series of amplifications, a fragment of the corrected size (497 bp) was generated from brain and

gonads of larvae and from testis tissue of pupae (Fig. 3.1). Clear amplification of this product was only observed after a PCR of 40 cycles (Fig. 3.1). In a second series of amplifications, however, carried out on an independent set of cDNAs, a truncated product of 414 bp was generated from pupal testis tissue after 40 cycles of PCR (Fig. 3.2). After sequencing of this product, differences at the 3rd exon were revealed when comparing with the cDNA sequence in Genbank and the 497 bp PCR product from the first set of independent cDNA samples (Fig. 3.3) (see also below).

Other primer pairs that were designed to amplify other parts of BmR2D2 mRNA, were unsuccessful (Table 3.1). It should also be noted that primer pair 5, that amplifies a sequence within the third exon, was successful using genomic DNA as template, but not using cDNA derived from DNase-treated RNA (Fig. 3.4), indicating functionality of the relevant primer pair. Moreover, PCR fragments from primers that cover the 3'-part of the R2D2 gene for 35-40 cycles, were shown to be non-specific after sequencing. Therefore, it is concluded that the BmR2D2 mRNA is either not expressed or only expressed at very low levels in the tissues of the *Bombyx* Daizo strain that was investigated.

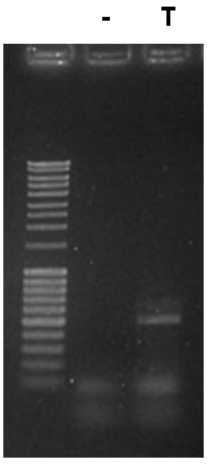


Fig. 3.2. Application of BmR2D2 for 40 cycles using an independent set of cDNAs. "-" indicated that the sample was a negative control which used MQ water in replacement of cDNA; "T" indicated the sample was amplified with larval testis cDNA.

3.3.4 Evidence for presence of mutant BmR2D2 genes

When the amplified fragment of 414 bp corresponding to the 5'-part of BmR2D2 mRNA (amplified from testis tissue in the second set of amplifications by primer pair 1, see above) was compared to the genome sequence of *Bombyx,* differences in the sequence of the third exon were revealed (Fig. 3.3). It was observed that a sequence of 83 nt was deleted from the sequence of the PCR fragment. Because this sequence is not flanked by consensus intron

splice recognition sequences (GT … AG), the most likely explanation must be that the exon has undergone a small deletion and therefore corresponds to a mutant allele of the *BmR2D2* gene.

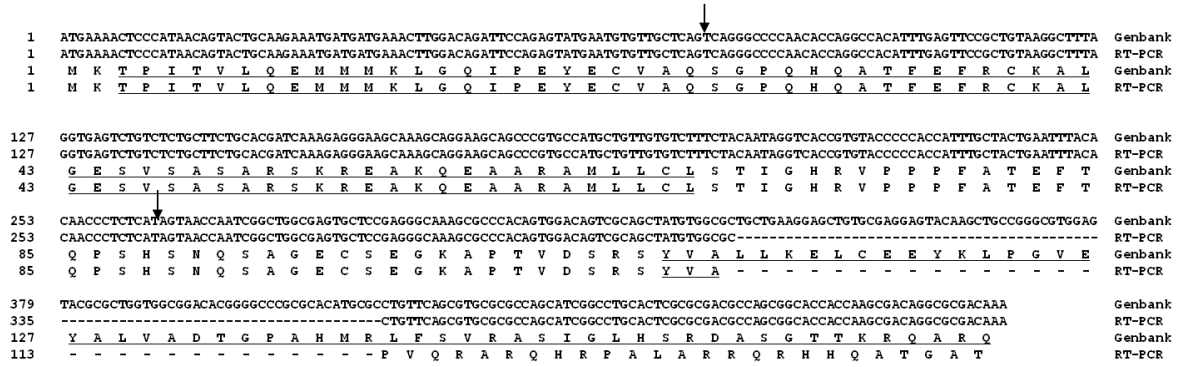


Fig. 3.3. Sequence of *BmR2D2* partial cDNA fragment generated by PCR using primer pair 1 (Table 3.1) from testis tissue (Daizo strain) and its comparison with the Genbank sequence of *BmR2D2* mRNA (NM_001195078). Splice sites are indicated by vertical arrows. The 83 bp deletion in the third exon that is absent in the amplified fragment is indicated as well as the frame-shift in the amino-acid sequence after the deletion. Sequences corresponding to the two dsRNA-binding domains are underlined.

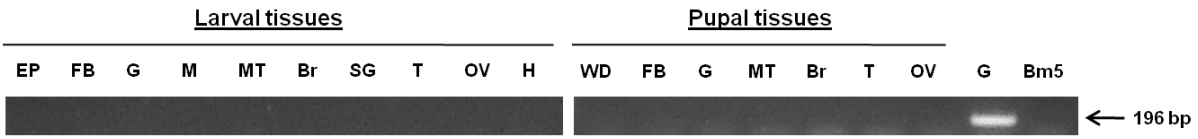


Fig. 3.4. Amplification of BmR2D2 for 35 cycles from tissues and Bm5 cells using primer pair 5. While no products are generated from cDNA samples, a clear amplification product of 196 bp was obtained from 300 ng of genomic DNA (lane "G"). Abbreviations: larval tissues: EP = epidermis; FB = fat body; G = midgut; M = thoracic muscles; MT = Malpighian tubules; Br = brain; SG = silk glands; T = testis; OV = ovaries; H = haemocytes; pupal tissues: WD = wing disk; FB = fat body; G = midgut; MT = Malpighian tubules; Br = brain; T = testis; OV = ovaries.

3.4 Discussion

In Lepidoptera, large differences in RNAi efficiency are observed among different species, developmental stages and even target genes (Terenius et al., 2011). It is understood that efficiency of RNAi can be influenced by many parameters, for instance expression levels of components of the core RNAi machinery, competence for uptake of dsRNA from the hemolymph or the gut content, and presence of dsRNA degrading enzymes in tissues.

Our first approach to gain insight in this complex phenomenon focused on the expression of components of the RNAi machinery in different tissues and at different stages of the insect. Studies were carried out using the silkmoth *Bombyx mori* because of the

availability of its genome sequence (Consortium, 2008; Mita et al., 2004; Xia et al., 2004) that allows easy identification and subsequent detection of expression of genes that encode factors of the core machinery of the siRNA and miRNA pathways (Moazed, 2009; Siomi and Siomi, 2009). Factors of the different pathways were looked at because it has been reported that different pathways overlap and can compete with each other (Tomari et al., 2007; Zhou et al., 2008a). Moreover, it was reasoned that high expression of factors of the miRNA pathway (primarily involved in development) might interfere with the efficient operation of the siRNA pathway (primarily involved in viral and genome defense).

A major conclusion of this survey is that the majority of genes of the core small RNA machinery are ubiquitously expressed, with only minor differences among different tissues (Fig. 3.1). Only *BmPasha*, which encodes a co-factor for the nuclear Dicer enzyme Drosha in the miRNA pathway, showed an obvious difference in mRNA expression among different tissues (Fig. 3.1).

The major finding of this expression study was the very low to absent expression of the mRNA that encodes the small dsRNA-binding protein R2D2 in all silkmoth tissues (Fig. 3.1). It is noted that only a partial cDNA clone of *Bombyx R2D2* , corresponding to its 5'-part, was obtained. Moreover, it was also observed that in some amplifications, the PCR product was characterized by a frameshift in the ORF. It is noted that the observed frameshift occurs at the beginning of the second dsRNA-binding domain which results in its deletion. Therefore it is likely that the function of the protein is considerably affected (Fig. 3.3). Thus, it appears that in the Daizo strain at least two alleles of *BmR2D2* exist, that they differ in their 5'-parts and of which one allele corresponds with the annotated sequence, while the other one is affected by a deletion. In addition, the 3'-parts of the ORF could not be amplified, using primers based on the annotated sequence. Rapid amplification of cDNA ends (RACE) to amplify 3'-end could be applied to isolate the coding sequence for the C-terminus of BmR2D2 in the Daizo strain. These experiments should determine whether a functional BmR2D2 protein can be encoded by the mRNA. However, it is obvious that the *BmR2D2* mRNA is expressed at very low levels (5'-part is only clearly detectable after 40 cycles of amplification) and therefore represents a limiting factor in the RNAi core machinery.

In *Drosophila*, where the action mechanism of the RNAi machinery has been investigated at the molecular level. It is shown that R2D2 is necessary for Dicer-2 to form the complex to enhance the RNAi efficiency. Researchers found that Dcier-2/R2D2 complex was much more effecitive than Dicer-2 alone to generate more siRNAs. Meanwhile,they found that Dcier-2/R2D2 complex associated more Ago2 proteins than the Dicer-2 alone or the

mutant complex (Liu et al., 2003). These experiments together indicate that Dcer-2/R2D2 not only generates siRNA from dsRNA but also binds to siRNA and ficilitates its loadint onto Ago-2, the central effector in the RISC complex involved in defense against transposable elements and viral infections, indicating that R2D2 bridges the initiation and effector steps of the *Drosophila* RNAi pathway by facilitating siRNA passage from Dicer to RISC (Liu et al., 2003; Saleh et al., 2006).

Chapter 4

Evaluation of the role of R2D2 for successful RNAi in silkmoth-derived Bm5 cells*

* Partially adapted from:

Luc Swevers, Jisheng Liu, Hanneke Huvenne, Guy Smagghe (2011). Search for limiting factors in the RNAi pathway in silkmoth tissues and the Bm5 cell line: the RNA-binding proteins R2D2 and Translin. PLoS One 6: e20250.

4.1 Introduction

RNAi is an evolutionarily conserved gene silencing pathway trigger by small RNAs, which target at the gene-specific mRNA (Siomi and Siomi, 2009). In *Drosophila*, the siRNA pathway is well clarified. Dicer-2 cleaves dsRNA into ~20-30 siRNA that are loaded into RISC that in turn cleaves mRNAs homologs to the siRNAs (Siomi and Siomi, 2009). In this process, R2D2, a dsRNA binding protein that acts as a co-factor of Dicer-2, functions as the bridge between the initiation (generation of siRNA from dsRNA) and effector (binding siRNA and facilitating its loading onto RISC) steps in the *Drosophila* RNAi pathway (Liu et al., 2003) which is also to help transfer the siRNA to Ago proteins (Siomi and Siomi, 2009).

R2D2 was firstly identified from the *Drosophila* derived S2 cells extracts in 2003 (Liu et al., 2003). Because this protein contains two dsRNA-binding domains (R2) and is associated with Dicer-2 (D2), it is named R2D2 (Fig. 4.1) (Liu et al., 2003). In the previous report, Dicer-2 and R2D2 could stabilize each other in the insect cells, where depletion of Dicer-2 by RNAi could diminish the protein level of R2D2, whereas RNAi of R2D2 caused a modest reduction in Dicer-2 protein in S2 cells (Liu et al., 2003). Moreover, recombinant Dicer-2 and R2D2 proteins were produced at much higher levels when expressed jointly than individually in insect cells (Liu et al., 2006). Although Dicer-2 is vital for siRNA generation, R2D2 is dispensable for siRNA production. In the meantime, R2D2 and Dicer-2 could coordinately bind the siRNA duplex and load siRNA onto RISC (Liu et al., 2006).

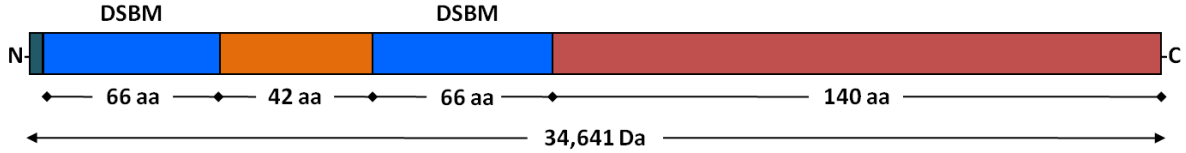


Fig. 4.1. Protein structure of BmR2D2. BmR2D2 contains two dsRNA-binding motifs (DSBM).

In *B. mori*, R2D2 was firstly reported in our previous study (Swevers et al., 2011). It is important to test if R2D2 could also affect the RNAi pathway in the silkworms. However, the expression of BmR2D2 is absent in most of the silkmoth tissues, except in the brain, testis and ovaries, and Bm5 cells (Chapter 4). Because our unsuccessful attempts to amplify the ORF of *Bombyx* R2D2 from tissues of the silkmoth strain that is cultured in our lab (Daizo), it was dicided to express the R2D2 homolog from *Tribolium castaneum* (TcR2D2; accession NM_001134953). In this chapter, we mainly focus on the RNAi response in the cells. Using tissue culture cells, two types of RNAi can be investigated, depending on the method of dsRNA administration. In case of intracellular RNAi, dsRNA is introduced into the cells with

high efficiency by transfection with a lipophilic reagent and the RNAi response can be thought to depend only on the competence of the core RNAi machinery in the cell. However, in some cell lines, such as *Drosophila* S2 cells, it is observed that simple "soaking" of the cells in growth medium supplemented with dsRNA at concentrations between 5-20 μg/ml can result in silencing effects (Saleh et al., 2006), which is through the endocytosis pathway prior to its presentation to the intracellular RNAi machinery.

4.2 Materials and methods

4.2.1 Expression and reporter constructs

To generate expression constructs for *Tribolium* R2D2 (TcR2D2), the ORF was amplified by PCR from *Tribolium* whole body using the primers 5'-ACTT*GGATCC*CAAC**ATG**TCCCGACAAAATACAAAAACC-3' (forward) and 5'-ACTT*GGATCC*CCCTTTGGCACCCTTGGTCATATAAA-3' (reverse). Both primers contain a BamHI cloning site (italic) that allows in-frame cloning with the C-terminal Myc-His sequence in the pEA-MycHis lepidopteran expression vector (Douris et al., 2006). The forward primer also contains a Kozak initiation sequence (underlined) and an ATG start codon (bold). PCR products were digested with BamHI and cloned in the corresponding site of the pEA-MycHis vector to generate the pEA-TcR2D2-MycHis expression vectors.

The reporter construct, pA-Luc, was constructed by subcloning the firefly luciferase ORF-SV40 poly(A) sequence from the pGL3 vector (Promega) as a HindIII-BamHI fragment downstream of the actin promoter in the pBmA expression plasmid (Johnson et al., 1992). The ecdysone-inducible reporter construct pERE-gfp was used to normalize for transfection efficiency (Swevers et al., 2004).

4.2.2 Generation of dsRNA

pLitmus 28i vectors (New England Biolabs) containing 0.3-0.7 kb fragments from the ORFs of firefly luciferase, GFP, BmCAP (Georgomanolis et al., 2009) and BmHR3 (Eystathioy et al., 2001) were linearized by EcoRI or HindIII and subjected to RNA synthesis reactions using T7 RNA polymerase according to the manufacturer's insructions (Fermentas). After digestion of the template DNA with RQ1 RNase-free DNase (Promega), synthesized ssRNA was purified by phenol-chloroform extraction/ethanol precipitation, re-suspended in annealing buffer (150 mM NaCl, 1 mM EDTA) and annealed with its complement by heating at 94ºC for 2 min followed by slowly cooling to room temperature.

4.2.3 Cell lines, transfections and RNAi functional assays

Bm5 cells (Grace, 1967) or Hi5 cells (Granados et al., 1994) were transfected according to established protocols (Johnson et al., 1992). For expression studies, 1.5 μg/ml of pEA-TcR2D2-MycHis expression vectors together with 1 μg/ml of pBmIE1 helper plasmid encoding the ie-1 gene for *B. mori* nuclear polyhedrosis virus (BmNPV) (Lu et al., 1997) was used.

For intracellular RNAi experiments, Bm5 cells were transfected with 0.9 μg/ml of pA-Luc, 0.2 μg/ml of pERE-gfp, 0.9 μg/ml of pEA-TcR2D2-MycHis and 1 μg/ml of nucleic acid (DNA, non-specific dsRNA or specific dsRNA at different ratios). Two days after transfection, RH-5992 (Rohm and Haas Co.) (Swevers and Iatrou, 1999) or chromafenozide (Sankyo Agro Co. Ltd) (Soin et al., 2010) was added at 200 nM to induce the ecdysone reporter gene (used as normalization for luminescence measurements).

In the experiments that evaluated RNAi by addition of dsRNA to the extracellular medium, Bm5 cells were transfected with 1.4 μg/ml of pA-Luc, 0.2 μg/ml of pERE-gfp and 1.4 μg/ml of pEA-PAC (negative control) or pEA-TcR2D2-MycHis. Before transfection, cells were incubated for two days with different concentrations of nucleic acid (DNA, non-specific dsRNA, specific dsRNA) at a concentration of 15 μg/ml. After transfection, cells were incubated with the same concentrations of nucleic acid for another two days. RH-5992 or chromafenozide was subsequently added at a concentration of 200 nM and luminescence or fluorescence in individual wells was quantified 24 hrs later.

Soluble cellular extracts of transfected cell populations were prepared as described above and directly used for fluorescence measurements or processed for luminescence measurements using the Steady-Glo® Luciferase Assay System kit (Promega) according to the manufacturer's instructions. Specific luciferase or fluorescence activities were calculated as luminescence/fluorescence or fluorescence/luminescence ratios. Both fluorescence and luminescence measurents were carried out with an Infinite M200 luminometer (Tecan) (Soin et al., 2010; Swevers and Iatrou, 1999).

4.2.4 Western blot analysis

Protein gel electrophoresis and Western blot analysis were carried out as described (Tsitoura et al., 2010). Transfected cells were collected by centrifugation and pellets were suspended in phosphate buffered saline (PBS; 100 μl per 10^6 cells). After freezing for 15 min at -70ºC, the cell suspension was subjected to high speed centrifugation (12000g, 15 min) and both supernatants (as soluble protein fraction) and cell pellets (as insoluble protein fraction)

were collected. Supernatants were diluted 1:1 (v:v) with cracking buffer while cell pellets were solubilised in 200 μl cracking buffer (Georgomanolis et al., 2009). Thirty μl of each fraction was loaded in individual lanes of protein gels. The antibodies that were used in Western blot analysis were mouse anti-Myc (Cell Signalling; at 1:1000) and rat anti-tubulin (Serotec; at 1:1000). Corresponding secondary HRP-conjugated anti-mouse, anti-rat (both Chemicon) and anti-guinea pig (Jackson) antibodies were used at 1:1000 (anti-mouse and anti-rat) or 1:5000 (anti-guinea pig). Pierce SuperSignal West Pico chemiluminescent substrate (ThermoScientific) was used for detection.

4.2.5 Immunofluorescence microscopy

Fluorescent staining of Bm5 or Hi5 cells with specific antibodies was carried out as described before (Labropoulou et al., 2008). For detection of Myc-tag fusion constructs, primary mouse anti-Myc and secondary FITC-labeled anti-mouse (Sigma) antibodies were both used at 1:200. The cells were stained with DAPI and finally mounted in Mowiol 4-88 (Sigma) and observed with a wide field Nikon TE 2000 fluorescence microscope.

4.2.6 Sequence comparison and phylogenetic analysis

Alignment of BmR2D2 (accession NM_001195078), DmR2D2 (AAF52561) and TcR2D2 (NP_001128425) was generated by MegAlign in DNAStar with ClustalW method (Thompson et al., 1994). Based on the multiple alignment, the phylogenetic tree was constructed and the sequence divergence was calculated.

4.3 Results

4.3.1 Low homology among R2D2s

Althought we did not amplify the ORF of *Bombyx* R2D2, another group in Japan have successfully cloned the ORF in another *Bombyx* strain. By using their published R2D2 mRNA sequence, we translated the nucleotide sequence into amino acid sequence. At the amino acid-level, sequence identities between *Bombyx* R2D2 and *Tribolium* and *Drosophila* R2D2 are 22% and 16%, respectively, while similarities amount to 30-39% (Fig. 4.2). The highest identities/similarities are found in the two dsRNA-binding domains (21-34% and 39-60% respectively). The phylogenetic tree also reveals that TcR2D2 and DmR2D2 are more closely clustered and form a subgroup, which diverges with BmR2D2 (Fig. 4.2).

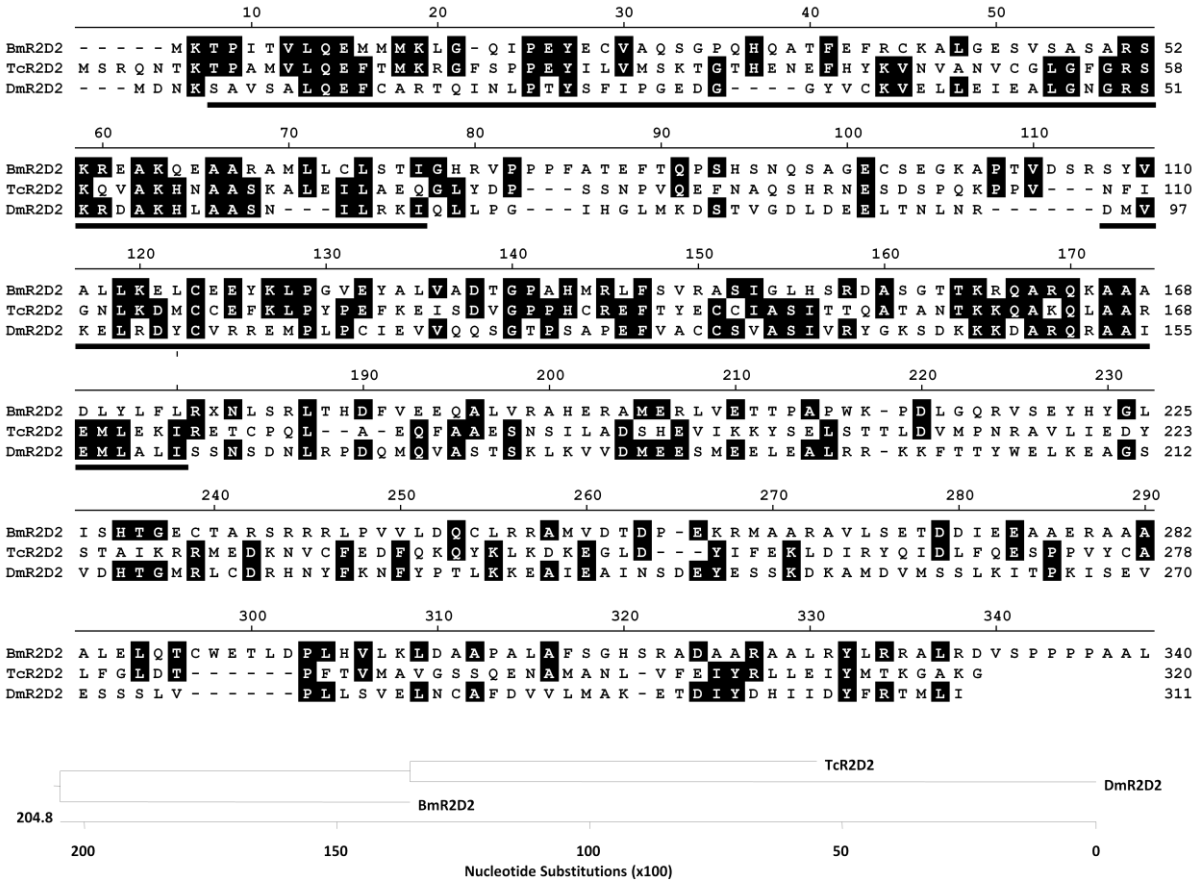


Fig. 4.2. Alignment of the amino-acid sequences of the R2D2 proteins from *Bombyx mori* (BmR2D2), *Tribolium castaneum* (TcR2D2) and *Drosophila melanogaster* (DmR2D2) and the construction of phylogenetic tree. Identical amino-acids are boxed in black. The two dsRNA-binding domains are underlined.

4.3.2 The intracellular RNAi response in Bm5 cells and the lack of stimulation by *Tribolium* R2D2

It was observed that intracellular RNAi occurs efficiently in Bm5 cells in the absence of BmR2D2 expression. Co-transfection of the actin-luciferase reporter with Luc-dsRNA results in a dose-dependent reduction of luciferase activity (Fig. 4.3). This response is specific, since a high dose of a non-specific dsRNA (BmCAP-dsRNA) did not result in a decrease in luciferase activity.

In *Drosophila*, genetic analysis has shown that the R2D2 protein is an important factor in the RNAi process through its role in the transfer of siRNAs to the Ago-2 effector protein (Liu et al., 2003). Because of its relative importance in the process of RNAi, at least in *Drosophila*, it was decided to focus on R2D2 to see whether its expression in Bm5 cells could stimulate the RNAi process in Bm5 cells.

Because our unsuccessful attempts (Chapter 3) to amplify the ORF of *Bombyx* R2D2 from tissues of the silkmoth strain that is cultured in our laboratory (Daizo), it was decided to express the R2D2 homolog of *Tribolium castaneum* (TcR2D2; accession NM_001134953).

The complete ORF of TcR2D2 was amplified from cDNA of *Tribolium* tissues, cloned in the pEA-MycHis lepidopteran expression vector and used in co-transfection experiments with luciferase reporter plasmid and dsRNA. However, it was observed that co-expression of TcR2D2 does not stimulate intracellular RNAi after transfection of dsRNA. As is shown in Fig. 4.3 (right panel), the dose-dependent decrease in luciferase activity was not altered during co-transfection with TcR2D2 expression plasmid. In contrast to control transfections, in the presence of TcR2D2 a small knockdown (~30%) was observed using non-specific dsRNA (Fig. 4.3, right panel). However, statistical analysis showed that the knockdown was not statistically significant (two-tailed student's *t*-test; P>0.05; N=3).

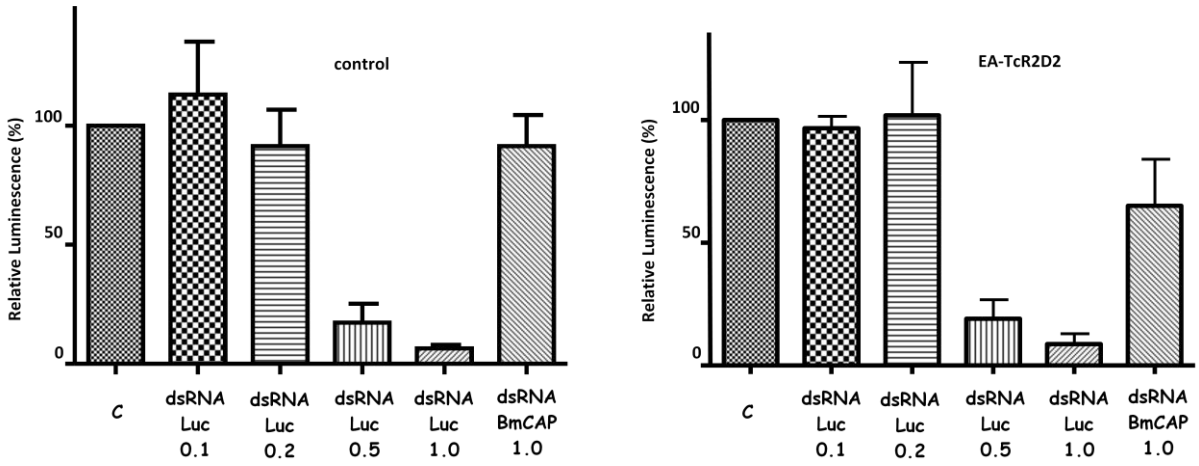


Fig. 4.3. Intracellular RNAi functional assays after transfection of dsRNA. Bm5 cells were transfected with DNA and either non-specific dsRNA or specific dsRNA at different ratios in the absence (Left) or presence (Right) of TcR2D2 expression plasmid. Indicated is the amount of luminescence in comparison with cells that received an equivalent amount of non-specific DNA (C; 100%). Experimental conditions include treatment with BmCAP-dsRNA and Luc-dsRNA at the indicated amounts (μg/ml in transfection mixture) (N = 3).

4.3.3 Absence of silencing in Bm5 cells after soaking with extracellular dsRNA and the lack of stimulation by *Tribolium* R2D2

One possible explanation for the deficiency in Bm5 cells to respond to dsRNA in the extracellular medium, could be the inability of its core machinery apparatus (lacking BmR2D2) to become activated by the small amounts of dsRNA that are transported from the extracellular medium to the cytoplasm. It was therefore investigated whether ectopic expression of R2D2 protein is capable to sensitize the core RNAi machinery to the

internalization of dsRNA and make the cell line competent to respond to extracellular dsRNA.

To test whether expression of TcR2D2 results in gene silencing following soaking with dsRNA, Bm5 cells were grown in culture medium containing Luc-dsRNA (or non-specific DNA or non-specific dsRNA) at different concentrations up to 10 μg/ml, a concentration sufficient to achieve efficient gene-knockdown in *Drosophila* S2 cells (Douris et al., 2006). The cells were subsequently transfected with a luciferase reporter together with the TcR2D2 expression construct (as well as the ERE-gfp reporter which was used for normalization). Transfected cells were then again 'soaked' with dsRNA for an additional period of three days before evaluation. However, ectopic expression of TcR2D2 did not result in silencing of luciferase activity following addition of Luc-dsRNA to the culture medium for a total period of 5 days (Fig. 4.4, left panel).

It has also been reported that RNAi can work more efficiently on induced genes versus constitutive genes, when dsRNA is added prior to the induction (Terenius et al., 2011). To investigate this possibility, GFP-dsRNA was added to Bm5 cells before and after transfection with an ERE-gfp reporter construct in the absence or presence of the TcR2D2 expression construct. After induction of the ecdysone reporter by 200 nM ecdysone agonist also in this case no significant gene knockdown was observed (Fig. 4.4, right panel).

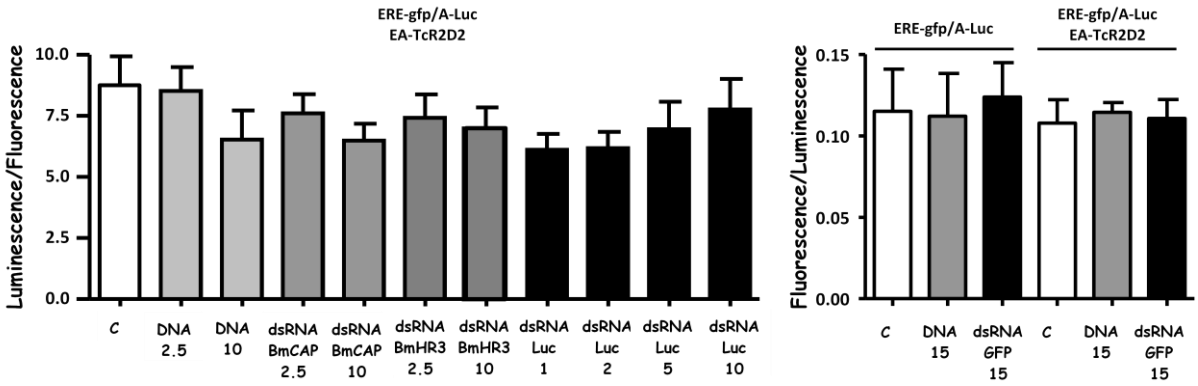


Fig. 4.4. Extracellular RNAi functional assays after addition of nucleic acid (DNA or dsRNA) to the tissue culture medium. Cells were soaked with extracellular dsRNA for two days before transfection and for three days after transfection. Experimental conditions include treatment with DNA, BmCAP-dsRNA, BmHR3-dsRNA, Luc-dsRNA and GFP-dsRNA at the indicated concentrations (in μg/ml). Left: Luciferase assays to evaluate expression of the constitutive A-Luc transgene. Indicated is the luminescence/fluorescence ratio (N = 3). Right. Relative fluorescence measurements (fluorescence/luminescence ratios) 24 hrs after induction of the ecdysone ERE-gfp reporter with 200 nM of ecdysone agonist. Cells were soaked with nucleic acid (DNA or dsRNA) for two days before transfection and for another three days after transfection. At two days after transfection, cells were induced with hormone agonist for a period of 24 hours (N = 3).

4.3.4 Accumulation of TcR2D2 at defined locations in the cytoplasm of tissue culture cells

Because high protein expression levels can be obtained in Hi5 cells following transfection (Douris et al., 2006), it was decided to use this cell line for the study of the subcellular localization of TcR2D2. Following transfection of the expression constructs for TcR2D2 protein, Western blot analysis indeed showed very high levels of expression of TcR2D2 (Fig. 4.5A).

Immunofluorescence shows that TcR2D2 is located at defined positions in the cytoplasm of Hi5 cells (Fig. 4.5B). Quantitative analysis revealed that, of cells that were stained for TcR2D2, approximately 40% showed one spot, 29% displayed two spots while another 25% showed three or more spots (N = 129). In a minority of cells, a crescent-type of staining was observed that was localized at one side of the cell (6%).

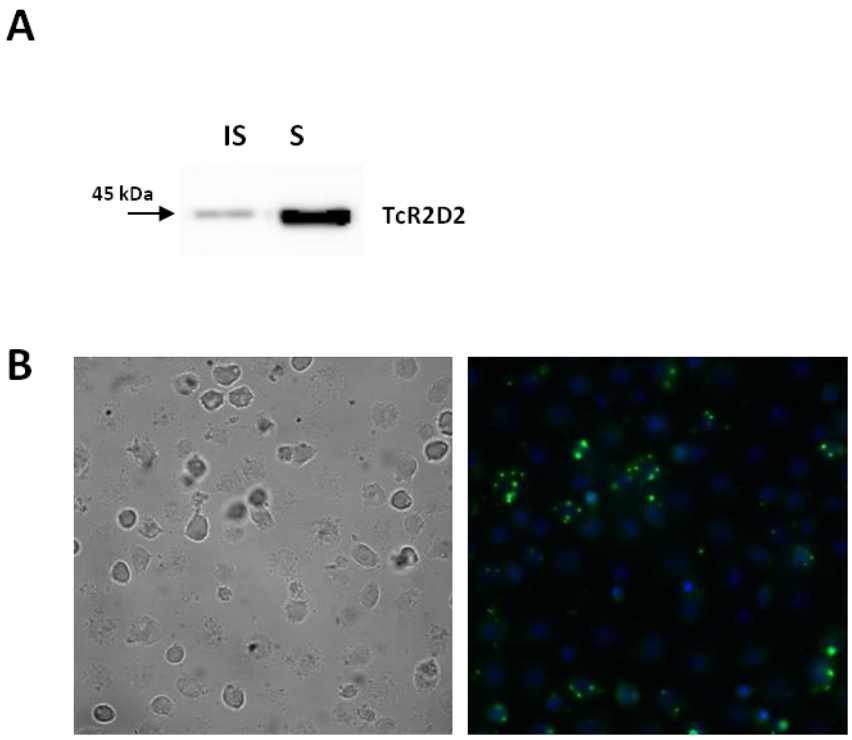


Fig. 4.5. Expression of myc-tagged TcR2D2 in transfected Hi5 cells. Panel A: Western blot analysis of expression of TcR2D2. Protein accumulates preferentially in the soluble fraction (S) of the cell extracts compared to the insoluble fraction (IS). Exposure times are 3 minutes. Panel B: Subcellular localization of myc-tagged TcR2D2 in transfected Hi5 cells compared to DAPI (Right). Corresponding bright field images are shown at the Left. Scale bar in Left Panel: 50 μm.

4.4 Discussion

In this chapter, the dsRNA binding protein R2D2 was studied to understand its involvement in the RNAi effect in Bm5 cells. Despite the absence of *R2D2* expression, it was observed that Bm5 cells could carry out specific gene silencing after transfection of dsRNA (Fig. 4.3). This indicates that BmR2D2 is not an essential component of the siRNA machinery in silkmoth cells. It is possible that another dsRNA-binding protein, Loquacious (Loqs), which is ubiquitously expressed in (Fig. 3.1), can compensate for the absence of BmR2D2, as was observed in *Drosophila* (Marques et al., 2010).

In contrast to transfection, plain addition of dsRNA to the culture medium of Bm5 cells ("dsRNA soaking") has no gene-silencing effect (Fig. 4.4), which has been also observed in other lepidopteran cell lines, such as Hi5 cell line from *Trichoplusia ni* (Beck and Strand, 2003) and UGA-CiE1 cell line from *Chrysodeixis* (Johnson et al., 2010). This observation contrasts with that of *Drosophila*-derived S2 cells where efficient RNAi is achieved when cells are "soaked" at doses between 5-20 μg/ml of dsRNA in the extracellular medium (Douris et al., 2006). This failure could be caused by the inability of the cells to internalize efficiently dsRNAs. However, studies have shown that fluorescently labelled dsRNA can be internalized by lepidopteran cells (Terenius et al., 2011) and it is therefore proposed that a functional barrier exists between internalization (which occurs by endocytosis in S2 cells (Saleh et al., 2006) and presumably in Bm5 cells as well) and presentation to the RNAi machinery in the cytoplasm or RNA-processing GW bodies.

Tribolium is a major model to study the role of genes in developmental processes of molting and metamorphosis by RNAi-mediated gene knockdown (Posnien et al., 2009). The process of systemic RNAi, achieved through injection of dsRNA into the haemocoel, is very effective in this species. *TcR2D2* mRNA is abundant in *Tribolium* tissues (unpublished results) and its high levels of expression are therefore correlated with the high efficiency of RNAi in this species. Therefore, Although sequence conservation among R2D2 proteins of different species is low, TcR2D2 has the same domain structure as BmR2D2 (Fig. 4.2) and may therefore enter the RNAi pathway in silkmoth cells.

We have tried to stimulate the RNAi efficiency in Bm5 cells in experiments that over-express *Tribolium* R2D2. However, ectopic expression of *TcR2D2* did not result in stimulation of specific gene silencing in both experiments of transfection or "soaking" with dsRNA (Fig. 4.3 & 4.4). As discussed before, RNAi efficiency may depend on other factors than R2D2 in Bm5 cells. On the other hand, it is also possible that *Tribolium* R2D2 is a suboptimal choice to complement for the absence of BmR2D2 in Bm5 cells. The similarities

between *Bombyx* R2D2 and *Tribolium* R2D2 are low (Fig. 4.2) and the staining patterns of these to proteins are different (Kolliopoulou and Swevers, 2013), there is a distinct possibility that TcR2D2 is not capable to form a functional complex with BmDicer-2 and participate in the RNAi process in Bm5 cells.

In a recent report, blocking of BmR2D2 in Bm5 cells did not cause any inhibitory effect to the dsRNA-mediated silencing. In the mean time, over-expression of BmR2D2 did not stiumuate dsRNA-mediated gene silencing in Bm5 cells. In this report, blocking of another dsRNA-binding protein BmLoqs which involved in the miRNA pathway did not inhibit the RNAi effect cells (Kolliopoulou and Swevers, 2013). These finding could be explained by two alternative mechanisms that may well act in parallel. The first explanation is that there exists another unknown dsRNA-binding protein that can carry out the function of BmR2D2 or BmLoqs in Bm5 cells. The second explanation is that there exists another alternative small RNA silencing pathway that acts in parallel with the siRNA pathway, which requires no dsRNA-binding protein. Hence, BmR2D2 is dispensable for RNAi pathway in Bm5 cells.

In summary, the experiments described in this work provide a first approach to the understanding of the efficiency of the RNAi response in the silkmoth and in Lepidoptera in general. Unexpectedly, due to the limited expression of R2D2 in the silkmoth tissues, we could not test whether *Bombyx* R2D2 could stimulate the RNAi efficiency or not; while the ectopic expression of *Tribolium* R2D2 proved not to enhance the RNAi effect. However, our study demonstrates that specific gene silencing occurs in silkmoth cells, if dsRNA is introduced efficiently in the cytoplasm of the cells by transfection. This process of specific gene silencing triggered by intracellular dsRNA occurs despite the absence of expression of *BmR2D2*. Since the formal demonstration that RNAi in Lepidoptera is working through the generation of siRNAs by a Dicer-2/R2D2 complex has not been published yet, it would also be valuable to isolate full-length BmR2D2 from appropriate silkmoth strains and test whether BmDicer-2/BmR2D2 dimers can generate siRNAs and load them to RISC as is observed for the DmDicer-2/DmR2D2 complex in purified S2 cell extracts (Liu et al., 2003; Liu et al., 2006).

Chapter 5

Expression of *Bombyx mori* DNA/RNA non-specific nuclease in lepidopteran insect culture cells*

* Adapted from

Jisheng Liu, Luc Swevers, Kostas Iatrou, Hanneke Huvenne, Guy Smagghe (2012). *Bombyx mori* DNA/RNA non-specific nuclease: expression of isoforms in insect culture cells, subcellular localization and functional assays. Journal of Insect Physiology 58:1166-1176.

5.1 Introduction

DNA/RNA non-specific alkaline nucleases have the interesting property to digest dsRNA and are therefore sometimes designated as double-stranded ribonuclease (dsRNase). However, the major characteristic of this class of enzymes is their broad substrate specificity. Homologs show highest activity towards dsRNA but are also able to digest ssRNA, ssDNA, dsDNA, as well as RNA/DNA hybrids (Meiss et al., 1999; Siwecka, 1997). Studies on homologs also indicated its involvement in mitochondrial DNA replication (Cote and Ruiz-Carrillo, 1993), DNA repair (Dake et al., 1988) and nucleic acid conformation (Nestle and Roberts, 1969; Robertson et al., 1968).

In the silkmoth, this enzyme was first detected in the digestive juice and midgut tissues (Mukai, 1965) and reported to be expressed in the midgut epithelium from where it was secreted into the gut lumen for digestion of nucleic acids (Arimatsu et al., 2007b). The enzyme was characterized as a Mg^{2+}-dependent alkaline nuclease degrading both DNA and dsRNA (Arimatsu et al., 2007a). Purification of the enzyme from the digestive juice and cloning of the gene indicated that the full-length protein consists of a signal peptide for secretion into the extracellular medium, a propeptide region which is cleaved off during maturation, and the mature, catalytically active, enzyme (Arimatsu et al., 2007b). It was determined that the enzyme is functional under alkaline conditions with its activity increasing in parallel to rising pH's (Arimatsu et al., 2007a). Thus, the enzyme is uniquely adapted to digest nucleic acids in the alkaline environment of the midgut of Lepidoptera.

Regardless of its role in digestion, however, it can be hypothesized that the enzyme also plays a role in the immune defense against viruses with dsRNA genomes, such as cypoviruses (Mori and Metcalf, 2010). In such case, as viral genomes in the extracellular medium are protected by the protein capsid shells and dsRNA replication intermediates are expected to accumulate in the cytoplasm after entrance into the cells, the enzyme needs to be expressed intracellularly. Conform to this hypothesis, it was found that expression of BmdsRNase (alkaline nuclease) is induced following infection with cypovirus, indicating a role in the innate immune response against viruses with dsRNA genomes (Wu et al., 2009). However, it is not known whether BmdsRNase can be expressed intracellularly and if it is capable to digest nucleic acids in the cytoplasm of the cells.

The expression of an enzyme with the capacity to degrade dsRNA, either as a secreted enzyme or with an intracellular function, could possibly also modulate the RNAi response that has evolved as defense against invasive nucleic acids. Triggers of such RNAi response consist of long dsRNAs that are processed by Dicer enzymes into siRNAs. siRNAs are

subsequently loaded into RISCs that scan RNAs for complementary sequences and trigger target cleavage (Carthew and Sontheimer, 2009; Siomi and Siomi, 2009). Recently, a major role of the RNAi pathway in the immune response against RNA viruses has been firmly established (Liu et al., 2011; Saleh et al., 2009; van Rij et al., 2006). Theoretically, dsRNase activity could have positive and negative effects on the RNAi response. On one hand, it can interfere with the RNAi process through degradation of both the long dsRNA triggers (both extra- and intracellularly) or the siRNA effectors (intracellularly). Alternatively, it can be involved in the non-specific degradation of invading nucleic acids after their specific recognition and cleavage by RISC complexes, hereby accelerating the removal of foreign nucleic acids.

5.2 Materials and methods

5.2.1 Experimental animals

The larvae of *B. mori*, Daizo strain, were reared on artificial diet (Yakuruto Co., Japan) at 25°C under a photoperiod of 12 h light and 12 h dark. Larval tissues were dissected from 5th instar larvae at day 4-5 after the molt. Tissues from 3-10 larvae were collected in eppendorf tubes on ice and samples were frozen at -70 ºC until further processing for RNA extraction. To isolate hemocytes, hemolymph was collected on ice after cutting the first proleg and subsequently centrifuged at low speed (800 g) at 4ºC to collect the cell pellet. The following tissues were used in the subsequent experiments: epidermis, fat body, midgut, thoracic muscles, Malpighian tubules, brain, silk glands, testis, ovaries and hemocytes.

5.2.2 RNA extraction, RT-PCR and 5'-RACE (rapid amplification of cDNA ends) PCR

Frozen tissues were homogenized in TRI Reagent (Sigma) and total RNA was extracted according to the manufacturer's protocol. The quantity of extracted RNA was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and/or by electrophoresis on 1% (w/v) agarose gels. First-strand complementary DNA (cDNA) synthesis was performed using a SuperScript II reverse transcriptase (Invitrogen, CA). Detailed RNA extraction and RT-PCR reactions were described by (Machado et al., 2007). Primers initially used for detection of *BmdsRNase* mRNA by PCR were 5'-GATCCGTCACCCCAATGTTG-3' (forward) and 5'-GCATTACCCACGAATCCAGC-3' (reverse). Control PCRs employed primers to amplify *Actin* mRNA (Machado et al., 2007). PCR optimization was performed with different cycle numbers and gradient temperature. Template cDNA was denatured at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s,

55°C for 30 s, and 72°C for 30 s for each cycle. A final elongation step was used at 72°C for 10 min. PCR products were run on 1.5% agarose gels and stained with ethidium bromide.

In order to confirm the expression of *BmdsRNase* mRNA in epidermis, fat body, muscle, Malpighian tubules, brain, and the silk glands, two sets of primers overspanning the whole ORF were used. The 5' fragment was amplified with ORF forward primer (5'-ATGCGTCTGACGCTTGTACT-3') and reverse primer (5'-GCATTACCCACGAATCCAGC-3') starting at 770 bp. The 3' fragment was amplified with forward primer (5'-AATGCCCTCGCAGACAGA-3') starting at 766 bp and ORF reverse primer (5'- TTATGTCAGAAGTCCGTTAACATTG-3'). Both PCR programs were performed as follows: 94°C denaturation for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s for each cycle, and a final elongation step at 72°C for 10 min.

5'-RACE was performed with SMARTer™ RACE cDNA Amplification Kit (Clontech, CA). 5'-RACE-Ready cDNA was generated from 1 µg RNA according to the user manual. The epidermis and midgut tissues were used in the PCR. MQ water (-) was used as a negative control. A gene specific reverse primer (5'-GAGTTGCCCGAAAGCACTGTTACC-3') was used in the first round PCR to amplify the 5'-RACE. PCR program was followed by 25 cycles of 68°C for 3 min. A nested reverse primer (5'-ACCGTCCCGACAGCAAGGTTAGA-3') was used in the second round to perform a nested PCR at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 60 s for each cycle, and a final elongation step at 72°C for 10 min.

5.2.3 Expression and reporter constructs

The complete ORF (with signal peptide and propeptide) of BmdsRNase was amplified with the forward primer 5'-CCAT*AGATCT*CAACATGCGTCTGACGCTTGTACT-3' and the reverse primer 5'-CCAT*AGATCT*TGTCAGAAGTCCGTTAACATTG-3', the PCR fragment was digested with BglII (in italics in the primer sequence) and cloned into the BamHI-site of the pEA-MycHis vector (Douris et al., 2006). The reverse primer is designed to allow the in-frame fusion with a MycHis-tag in the expression vector, to generate plasmid pEA-BmdsRNase(full)-MycHis. The same reverse primer was also used in combination with the forward primers 5'-AATT*AGATCT*CAACATGCTTCGAACGGATCTACCAGAG-3' and 5'-AATT*AGATCT*CAACATGAGCGGTTGCACATTCCGAGTC-3' to generate the expression plasmids pEA-BmdsRNase(pro)-MycHis and pEA-BmdsRNase(cat)-MycHis, which express

tagged versions of the BmdsRNase propeptide (signal peptide deleted) and the BmdsRNase processed/catalytic/mature domain (signal peptide and propeptide deleted) (Fig. 5.2).

To create BmdsRNase constructs with an alternative signal peptide sequence, the propeptide region or catalytic domain of BmdsRNase were amplified by forward primers 5'-AATT*GCATGC*TTCGAACGGATCTACCAGAG-3' or 5'-AATT*GCATGC*CAAGCGGTTGCACATTCCGAGTC-3', respectively, in combination with the common reverse primer described above and the PCR fragment was subsequently cloned in-frame with a chorion signal peptide (SP16) at the N-terminus (using the SphI-site, in italics in the primer sequences) (Farrell et al., 2000) and a MycHis-tag at the C-terminus (using the BglII-site), to generate the expression vectors pEA-SP16-BmdsRNase(pro)-MycHis and pEA-SP16-BmdsRNase(cat)-MycHis (Fig. 5.3).

To generate expression construct for Dicer-2, the complete ORF was amplified with the primers 5'-AATT*AGATCT*CAACATGACTACAACAGAAGGAATACAG-3' and 5'-AATT*AGATCT*GAATTAAGAGTCTAATATTTTGAG-3' which contain BglII-sites for cloning in-frame with the N-terminal Flag tag of a modified pEA vector (Douris et al., 2006). PCR conditions were 40 cycles, consisting of 94°C for 45 s, 55°C for 45 s, and 72°C for 5 min.

5.2.4 Cell lines and transfections

Hi5 (Granados et al., 1994) and Bm5 cells (Grace, 1967) were transfected according to established protocols (Johnson et al., 1992). For expression studies, 2 μg/ml of pEA-MycHis expression vector (described above) together with 1 μg/ml of pBmIE1 helper plasmid encoding the *ie-1* gene for *B. mori* nuclear polyhedrosis virus (BmNPV) (Lu et al., 1997), was used. Cells were harvested at 2-3 days after transfection for expression analysis or functional assays.

5.2.5 Protein extracts preparation

Transfected cells were centrifuged at 800 g for 5 min to separate the cell pellets and culture medium. The cell culture medium was used for secreted protein analysis, while the cell pellets were suspended in phosphate-buffered saline (PBS; 100 μl per 10^6 cells). Then this cell suspension was frozen for 15 min at -70ºC and subjected to high speed centrifugation at 12000 g for 10 min. After centrifugation, supernatants, containing cytosol and small cell organelles, and pellets, containing large cell organelles, membranes and chromatin, were collected. Both cell culture medium and supernatants were diluted 1:1 (v:v) with cracking

buffer while the pellets were solubilised in 200 µl of cracking buffer (0.125 M Tris (pH 6.8), 5% β-mercaptoethanol, 2% SDS, 4 M urea; (Georgomanolis et al., 2009). Protein concentration was measured with Coomassie Protein Assay Reagent Kit (Pierce).

5.2.6 SDS-PAGE and western blot analysis

Protein gel electrophoresis and Western blot analysis were carried out as described (Artuch et al., 2003). Ten micrograms of proteins were heated with 4 × NuPAGE LDS Sample Buffer (Invitrogen, CA) at 70ºC for 10 min according to the instructions (Invitrogen). SDS-PAGE was run with 10% Bis-Tris NuPAGE Gel (Invitrogen) in MOPS Running Buffer (Invitrogen).

For Western blot analysis, proteins were immediately transferred to PVDF membrane (Invitrogen). The membrane was blocked with 5% non-fat milk. For the BmdsRNase expression constructs, the first antibody used was mouse anti-Myc at 1:1000 (Cell Signalling, MA). The secondary HRP-conjugated anti-mouse antibody (KPL) was used at 1:1000. Then, 3,3'- diaminobenzidine tetrahydrochloride hydrate (Sigma) was used as substrate for development. For detection of Flag-tagged Dicer-2, membranes were first incubated with rabbit anti-Flag antibody (Sigma) at 1:1000 and subsequently with HRP-coupled anti-rabbit antibody. Pierce SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was used for detection.

5.2.7 dsRNA synthesis

pGEM-T vector (Promega) containing a green fluorescent protein (GFP) fragment was linearized by NcoI (Fermentas). T7 promoter was added to GFP fragment by PCR with the forward primer 5'- TAATACGACTCACTATAGGGTACGGCGTGCAGTGCT and reverse primer 5'- TAATACGACTCACTATAGGGTGATCGCGCTTCTCG (T7 promoter sequence is underlined). PCR conditions were 94°C for 2 min, followed by 5 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 70 s, and another 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 70 s. A final elongation step was used at 72°C for 3 min. dsGFP synthesis using the PCR fragment as template was carried out with MEGAscript RNAi kit (Applied Biosystems, CA).

5.2.8 Nuclease degradation analysis

In the DNA cleavage analysis, 1 x 10^6 Hi5 cells were transfected with 2 µg/ml of pEA-MycHis expression vector or pEA-Luc vector (control), together with 1 µg/ml of

pBmIE1 helper plasmid. Total DNA was extracted at 24 h, 48 h, and 96 h and 7 days after transfection. Fifty ng of DNA was used as template to perform PCRs with forward primer (5'-CGTGTCGCCCTTATTCCCTT -3') and reverse primer (5'-CACGCTCGTCGTTTGGTATG -3') to amplify plasmid DNA sequences. PCR procedure was at 94°C 30s, 55°C 30s, 72°C 30 s, 20 cycles.

In the dsRNA cleavage analysis, 1 x 10^6 Hi5 cells were co-transfected with 2 μg/ml of pEA-MycHis expression vector and 1 μg/ml of dsGFP, together with 1 μg/ml of pBmIE1 helper plasmid. The negative control cells were co-transfected with pEA-Luc and dsGFP, with the helper plasmid pBmIE1. Total RNA was extracted at 24 h, 48 h, and 72 h after transfection. First strand cDNA from 500 ng RNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and primer (5'-CTACCCCGACCACATGAAGC-3' – specific to dsGFP) at following program: 25 °C for 10 min, 42 °C for 1 h. PCR was performed with the forward primer (5'-GCTTCTCGTTGGGGTCTTTG-3') and reverse primer (5'-TCCAGGAGCGCACCATCTTC-3'). PCR procedure was at 94°C 30 s, 55°C 30 s, 72°C 25 s, 30 cycles. Amplification of the actin gene was carried out as internal control (Machado et al., 2007).

Quantification of PCR fragments in agarose gel was carried out by Quantity One 4.6.2 software (Bio-Rad, CA). Quantities of PCR fragments corresponding to the housekeeping gene actin were used for normalization. Relative expression levels of the genes were compared with their levels at 24 h after transfection (set as 100%). Statistical analysis of differences in normalized expression levels of each gene was carried out by a two-way ANOVA analysis.

5.2.9 RNAi response assay in Hi5 and Bm5 cell cultures

RNAi experiments were carried out as described before (Swevers et al., 2011). Hi5 or Bm5 cells were transfected with 0.9 μg/ml of pA-Luc luciferase reporter, 0.2 μg/ml of pERE-gfp fluorescence reporter, 0.9 μg/ml of expression plasmid (pEA-PAC (control; expresses puromycin N-acetyl transferase), pEA-BmdsRNase(full)-MycHis, pEA-BmdsRNase(pro)-MycHis or pEA-BmdsRNase(cat)-MycHis) and 1 μg/ml of nucleic acid (0.9 μg/ml DNA and 0.1 μg/ml dsLuc dsRNA (derived from luciferase ORF) (Swevers et al., 2011). Two days after transfection, the ecdysone agonist RH-5992 (Rohm and Haas) was added at 200 nM to induce the ecdysone reporter gene (used as normalization for luminescence measurements). Soluble cellular extracts of transfected cell populations were prepared as described (Swevers et al., 2011) and directly used for fluorescence measurements or processed for luminescence

measurements using the Steady-Glo® Luciferase Assay System kit (Promega) according to the manufacturer's instructions. Specific luciferase activities were calculated as luminescence/fluorescence ratios. Both fluorescence and luminescence measurements were carried out with an Infinite M200 luminometer (Tecan) (Soin et al., 2010). Statistical analysis of differences in normalized luciferase activity was carried out by a two-tailed Student's *t*-test.

5.2.10 Immunofluorescence microscopy

Immunofluorescent staining of Hi5 cells with specific antibodies was carried out as described before (Labropoulou et al., 2008). In brief, cells were fixed with 4% formaldehyde in PBS for 20 min and subsequently permeabilized with PBS supplemented with 0.1% v/v Triton X-100 (PBS-T) for 10 min. Cells were stained overnight at 4°C with mouse anti-myc antibody (Cell Signalling) or rabbit anti-Flag antibody (Sigma) at 1:200. Following five washes in PBS, FITC-conjugated goat anti-mouse secondary antibody (Sigma; at 1:200) or AlexaFluor-labeled anti-rabbit antibody (Molecular Probes; at 1:500) were added for 1 h at room temperature. Cells were again extensively washed with PBS, stained with DAPI (1 μg/ml, 5 min), mounted in Mowiol 4-88 (Sigma) and examined under a fluorescence microscope (Zeiss Axiovert 25 inverted microscope). For confocal microscopy, the cells were treated as described above and observed in a Bio-Rad confocal microscope (MRC 1024 ES) equipped with Lasersharp software (Bio-Rad) and a kryptonargon laser. Confocal microscope data were processed using the GNU Image Manipulation Program (GNU Project, http://www.gnu.org/).

5.2.11 Sequence comparison and phylogenetic analysis

Alignment of dsRNase of *B.mori* (NP_001091744), dsRNase of *Mamestra configurata* (AEA76311), putative dsRNase of *Spodoptera frugiperda* (CAR92521), *S. littoralis* (CAR92522) and *Danaus plexippus* (EHJ64029) was generated by MegAlign in DNAStar with ClustalW method (Thompson et al., 1994).

Other dsRNase homologs were found in GenBank, including alkaline nucleases from *D. plexippus* (EHJ75678), *Culex quinquefasciatus* (XP_001858177) and *C. quinquefasciatus* (XP_001844830), *Aedes aegypti* (deoxyribonuclease I, XP_001651912), *Anopheles gambiae* (XP_001687840), *A. darlingi* (hypothetical protein, EFR29824), *Drosophila pseudoobscura* (GA17708, XP_001352914), *D. persimilis* (GL22028, XP_002026571), *D. sechellia* (GM14909, XP_002042640), *D. ananassae* (GF10795, XP_001958179), *D. willistoni*

(GK12612, XP_002068162), *D. yakuba* (GE22538, XP_002095672), *D. melanogaster* (CG3819, NP_649078), *D. erecta* (GG13440, XP_001973287), *Glossina morsitans* (putative endonuclease, ADD19592), *Camponotus floridanus* (hypothetical protein, EFN63586), *Daphnia pulex* (hypothetical protein, EFX68769), *Paralithodes camtschaticus* (duplex-specific nuclease, BAH02823), *Penaeus monodon* (DNA/RNA non-specific endonuclease, ABF69938), *Marsupenaeus japonicus* (deoxyribonuclease I, CAB55635) and *T. castaneum* (deoxyribonuclease I, XP_973587).

A paralog of BmdsRNase was also found in the *Bombyx* genomic database (KAIKOBLAST, http://kaikoblast.dna.affrc.go.jp/) after carrying out tBLASTn search using the complete ORF of BmdsRNase as query. Complete ORF sequence of the paralog was tentatively predicted by taking the first upstream in-frame start codon (ATG) and the first downstream in-frame stop codon (TAA) in the genome sequence. Sequence information is presented in supplementary text 1. All the homologs and paralog mentioned above were used to construct a phylogenetic tree by MEGA4 and the bootstrap test was performed using Neighbor-joining method (Tamura et al., 2007).

5.3 Results

5.3.1 Broad expression pattern of BmdsRNase mRNA in different larval tissues

The expression of *BmdsRNase* mRNA in different tissues and hemocytes from 5th instar larvae at day 4-5 after molting was analyzed. As it shows in Fig. 5.1A, a 420 bp fragment was amplified from cDNAs not only of midgut tissue but also from other tissues such as epidermis, fat body, thoracic muscles, Malpighian tubules, brain, and the silk glands. Sequencing showed that this fragment corresponds to *BmdsRNase* mRNA (Genbank accession number NM_001098274) (Arimatsu et al., 2007b). A higher MW fragment of 613 bp was amplified from cDNA of testis, ovaries and hemocytes which was shown to correspond to the genomic sequence of BmdsRNase flanked by the primers (data not shown); it is assumed that this fragment corresponds to amplification of either non-spliced mRNA or traces of genomic DNA that may have contaminated the RNA preparation.

To confirm the expression of *BmdsRNase* mRNA in epidermis, fat body, thoracic muscles, Malpighian tubules, brain, and the silk glands, RT-PCR analysis was performed using two pairs of primers that overspan the complete ORF of *BmdsRNase*. As presented in Fig. 5.1B, 5'-fragment of 770 bp as well as 3'-fragment of 585 bp could be amplified from the larval tissue extracts. Sequencing results proved that they corresponded exactly with *BmdsRNase* mRNA in NCBI database (Genbank accession number NM_001098274).

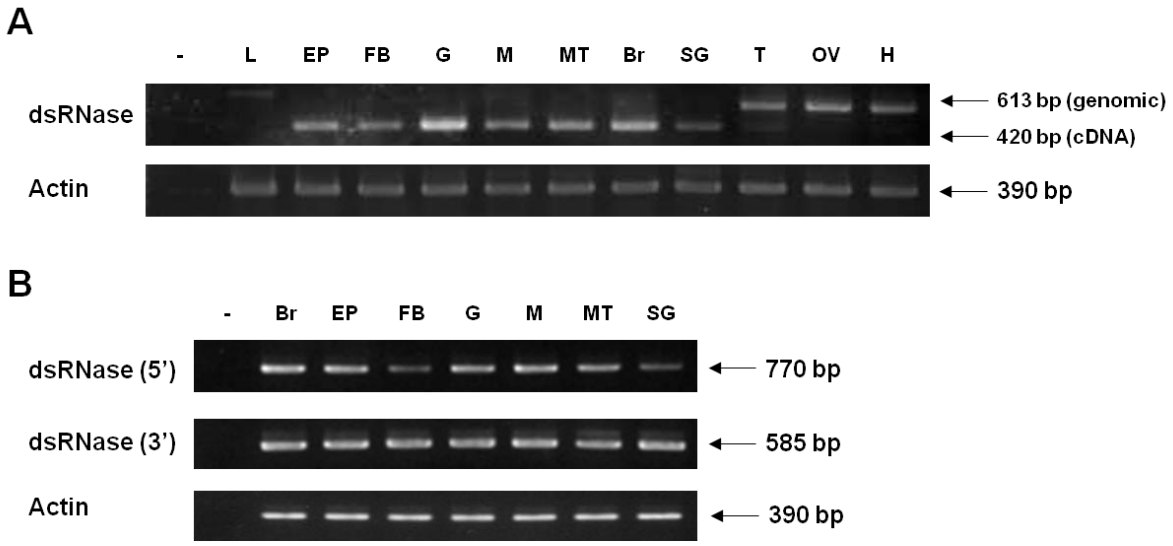


Fig. 5.1. *In vivo* expression pattern study of *BmdsRNase* mRNA in different larval tissues. Fragments were obtained after 35 cycles of PCR. Larval tissues, epidermis (EP), fat body (FB), midgut (MG), thoracic muscles (M), Malpighian tubules (MT), brain (BR), silk glands (SG), testis (T), ovaries (OV) and hemocytes (H), were collected from 5th instar larvae at day 4-5 after the molt. (A) A fragment of 420 bp was amplified from cDNAs in EP, FB, MG, M, MT, BR and SG; while a higher MW fragment of 613 bp was amplified from T, OV and H. Lane "L" shows amplification from 300 ng of DNA from a library of silkmoth vitellogenic follicles (Swevers et al., 1995). (B) Two fragments spanning the ORF of BmdsRNase were amplified from BR, EP, FB, G, M, MT and SG. Amplifications of 5'- and 3'-parts are shown in the upper and lower panels, respectively. "-" shows amplifications in the absence of template cDNA.

5.3.2 Expression constructs for full-length BmdsRNase and its derivatives

Using RNA from whole larvae of the silkmoth, the complete ORF of *BmdsRNase* was obtained by RT-PCR. The ORF of *BmdsRNase* encoded a 51 kDa precursor protein which could be divided into three domains, a signal peptide of 20 amino acids, an N-terminal propeptide of 45 amino acids and a mature peptide of 384 amino acids which is 43 kDa (Fig. 5.2A). In order to investigate the secretion and function of BmdsRNase, pEA-based expression vectors (Douris et al., 2006) were constructed.

The native version of the precursor protein is called BmdsRNase(full) and it has all the three domains (Fig. 5.2A). This form was not secreted by transfected Hi5 cells (data not shown), which prompted the construction of four other expression constructs. By the deletion of the signal peptid), the construct expressing BmdsRNase(pro) was generated (Fig. 5.2A). A final construct was used to express the processed/catalytic/mature BmdsRNase peptide, corresponding to the form as isolated from the digestive juice, named BmdsRNase(cat) (Fig. 5.2A).

By substitution of the native signal peptide with a heterologous signal peptide,

derived from silkmoth chorion proteins (SP16), another form of BmdsRNase called SP16-BmdsRNase(pro) was created (Fig. 5.3A). By the deletion of the propeptide from SP16-BmdsRNase(pro), a constructs expressing SP16-BmdsRNase(cat) was generated (Fig. 5.3A). All the constructs have a MycHis-tag at the C-terminus (Fig. 5.2A & 5.3A).

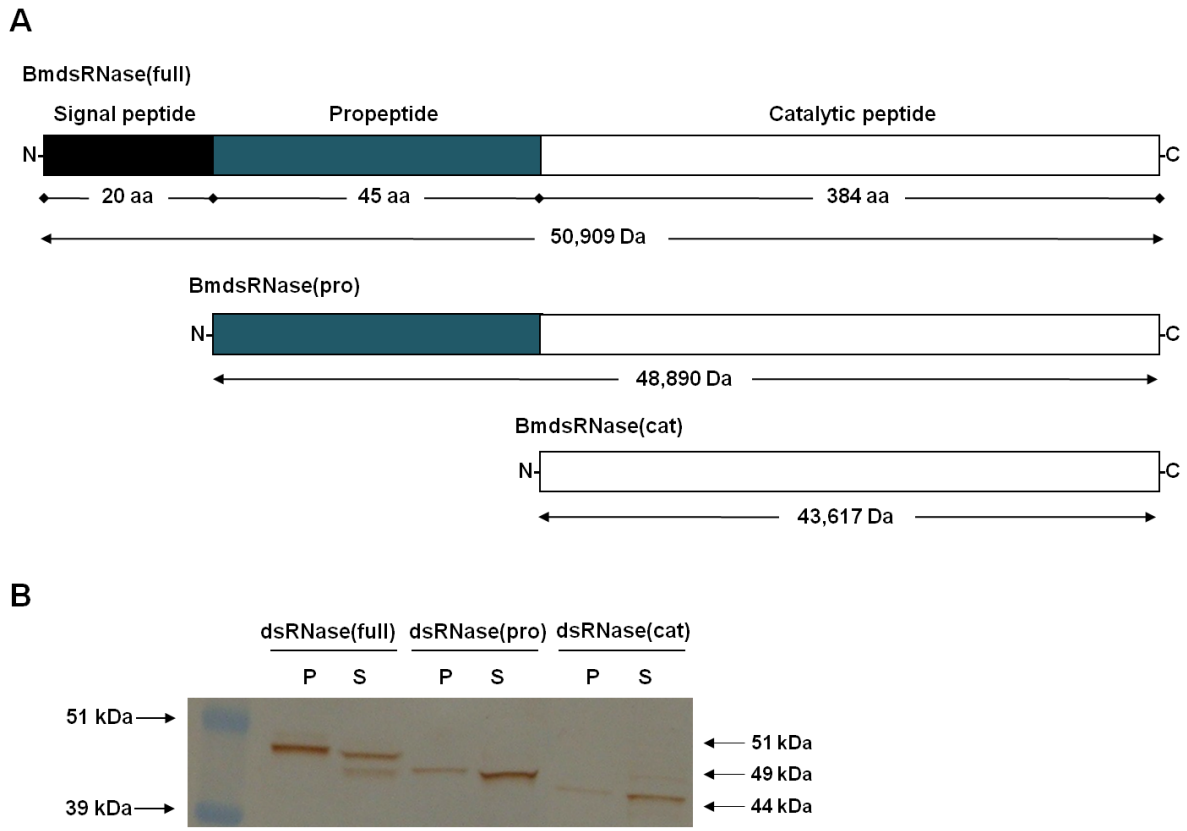


Fig. 5.2. Expression of different forms of BmdsRNase in Hi5 cells. (A) Structures of BmdsRNase expression constructs. The full structure of BmdsRNase has three domains, a signal peptide, an N-terminal propeptide and a catalytic/mature peptide. Construct with full structure is named BmdsRNase(full). Deletion of the signal peptide from the full construct results in the creation of BmdsRNase(pro). Construct only with catalytic domain is BmdsRNase(cat). (B) Western blot analysis of BmdsRNases in pellets (P) and supernatants (S) of Hi5 cell extracts. The different forms of BmdsRNase accumulated in both cell supernatants and pellets, albeit with different levels. It is noted that the MWs of the proteins in the pellets were a little higher than those in supernatants.

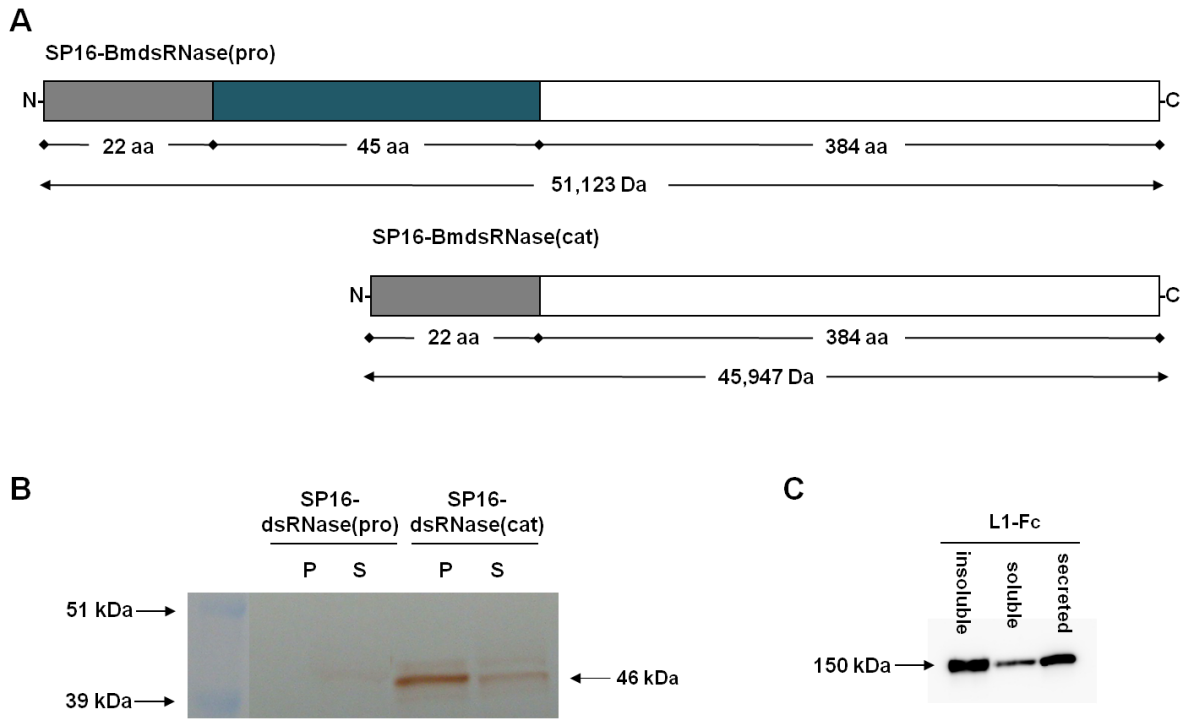


Fig. 5.3. Expression of different forms of BmdsRNase with alternative signal peptide in Hi5 cells. (A) Structures of SP16-BmdsRNase(pro) and SP16-BmdsRNase(cat) expression constructs. (B) Expression of SP16-BmdsRNase(pro) and SP16-BmdsRNase(cat) in Hi5 cells. Expression of SP16-BmdsRNase(cat) could be detected in the cellular pellets (P) and supernatants (S) by Western blot. (C) Efficient secretion of the mammalian L1-Fc protein to culture medium (M) with its native signal peptide.

5.3.3 BmdsRNase and its derivatives are expressed in Hi5 cell supernatants and pellets

Because the protein expression levels in Hi5 are high after transfection (Douris et al., 2006), it was decided to use this cell line to study expression in the Western blot analysis (Fig. 5.2B). Both expression and secretion of the BmdsRNase constructs were examined, by checking supernatants and pellets as well as the culture medium of the transfected cells.

Western blot analysis indicated that the three BmdsRNase forms were highly expressed in Hi5 cellular extracts (Fig. 5.2B). However, it was striking that none of the expression constructs of BmdsRNase with a signal peptide resulted in secretion of the protein (data not shown). For the two constructs without signal peptide, expressing BmdsRNase(pro) and BmdsRNase(cat), preferential expression in supernatants was observed (Fig. 5.2B). However, as observed in Fig. 5.2B, there were some differences in the MWs between proteins in the supernatants and pellets. MWs of the proteins in the pellets were slightly higher than those in supernatants for BmdsRNase(full) and BmdsRNase(pro).

In another report, efficient secretion of the enzyme juvenile hormone esterase fused to chorion signal peptide (and with its native signal peptide deleted) was observed (Farrell et al.,

2000). As shown in Fig. 5.3C, in a simultaneous set of transfections, efficient secretion of the mammalian L1-Fc protein was also observed. Therefore, we decided to substitute the native signal peptide of BmdsRNase with the chorion signal peptide (SP16). However, secretion of BmdsRNase proteins to culture medium was still not detected (data not shown). Expression could be detected in the cellular extracts which expressed SP16-BmdsRNase(cat) (Fig. 5.3B).

Because high expression levels were observed for BmdsRNase(full), BmdsRNase(pro) and BmdsRNase(cat), these three constructs were used in the subsequent experiments.

5.3.4 Over-expression of BmdsRNase results in increased DNA and dsRNA degradation in Hi5 cells

Functional assays of BmdsRNase activity have thus far only be performed in *in vitro* assays using purified mature BmdsRNase (43 kDa form) from the midgut juice (Arimatsu et al., 2007a,b). It is not known whether the incompletely processed forms have catalytic activity and if they are capable to digest nucleic acids when expressed intracellularly, as is the case in transfected Hi5 cells. It was therefore of interest to express the three isoforms in Hi5 cells and evaluate their catalytic activity towards co-transfected nucleic acids.

First, because all the pEA-BmdsRNase constructs over-express BmdsRNase in the cells, these over-expressed BmdsRNase proteins might also digest the DNA of the pEA vectors in the cells. To check this possibility, Hi5 cells were transfected with pEA-BmdsRNase expression vectors. After transfection, a fragment from the ampicillin region in the plasmid was amplified by PCR to detect the presence of the expression plasmids. Cells transfected with empty pEA vector were used as a negative control. From the PCR results it was apparent that all the vectors have been degraded after one week, due to endogenous nuclease activity in the cells (Fig. 5.4A). Only samples from cells transfected with BmdsRNase(cat) expression plasmid showed a significant increase in DNA cleavage at 48 h and 96 h after transfection (Fig. 5.4A). Quantification analysis showed that there were no significant differences among control cells, or cells transfected with BmdsRNase(full) or BmdsRNase(pro) (Fig. 5.4C). Only BmdsRNase(cat) vectors caused stimulation of degradation after 48 h ($P<0.05$) compared with 24 h. At 96 h, relative quantity of BmdsRNase(cat) vector DNA was reduced to 41%, while for the other samples vector DNA remained intact for >82% (Fig. 5.4C).

Similar method was used for the dsRNA cleavage experiment. pEA-BmdsRNase vectors were co-transfected with dsGFP and the quantity of dsGFP was examined by RT-

PCR. Judging from the RT-PCR results, BmdsRNase(pro) could not degrade dsGFP (as well as control cells). But both BmdsRNase(full) and BmdsRNase(cat) could digest dsGFP to a different extent (Fig. 5.4B). Although BmdsRNase(cat) was more active in the degradation of dsGFP (Fig. 5.4D; 52% and 26% of dsGFP could be detected at 48 h and 96 h, respectively), clear activity of BmdsRNase(full) to degrade dsRNA was also observed (for co-transfected dsGFP, 73% and 49% were present at the same time points). By contrast, in control cells, intact dsGFP amounted to 90% at 48h and 74% at 96 h.

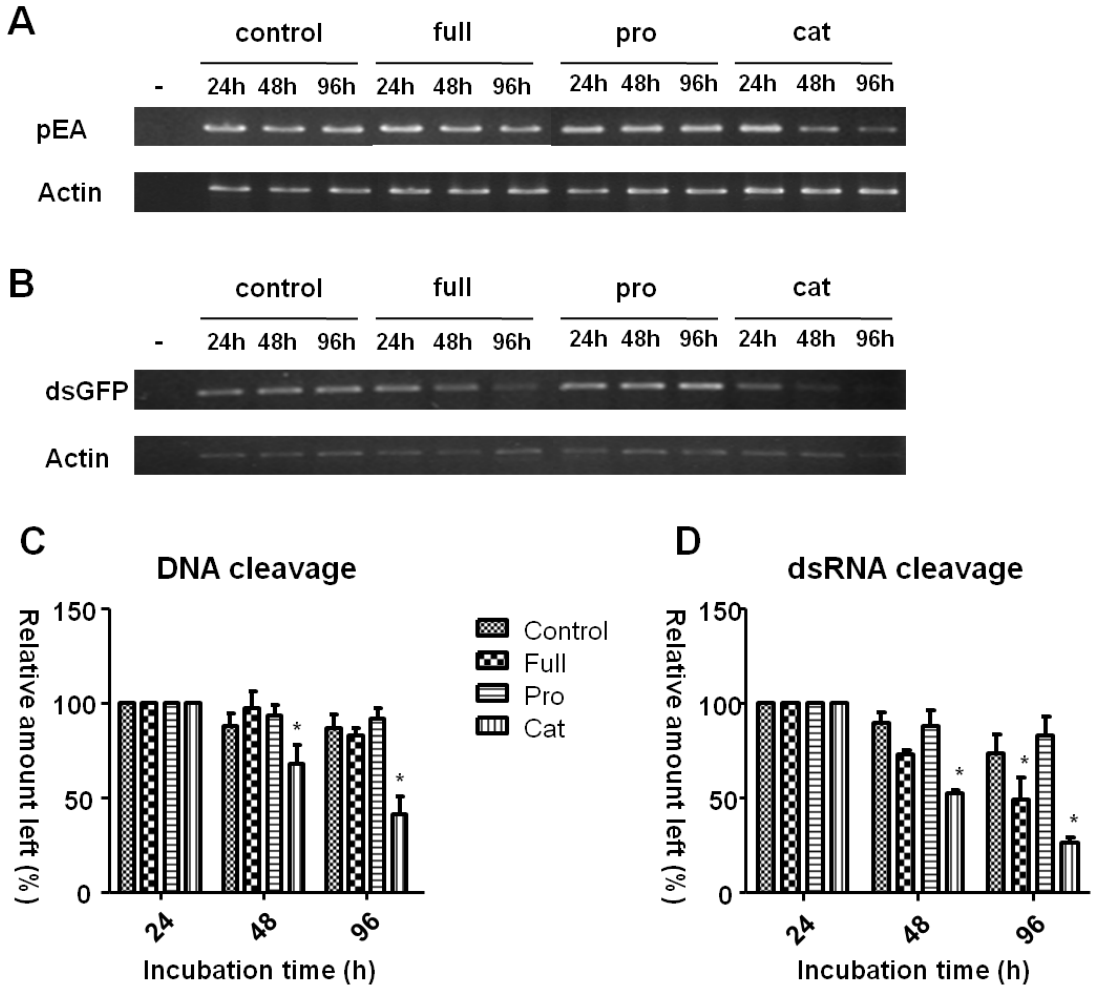


Fig. 5.4. BmdsRNase cleavage assay. (A) Hi5 cells were transfected with different pEA-MycHis expression vectors. Those cells transfected with pEA-Luc vector were used as control. Total DNA was extracted at 24 h, 48 h and 96 h after transfection. Fifty ng of DNA were used as template to perform PCRs to detect the presence of plasmid DNA. (B) Hi5 cells were co-transfected with different pEA-MycHis expression vectors and dsGFP. While the control was co-transfected with pEA-Luc and dsGFP. Total RNA was extracted at 24 h, 48 h and 96 h after transfection. Five hundred ng of RNA was used to perform RT-PCR to detect the presence of dsGFP. "-" shows amplifications in the absence of template cDNA. (C) and (D) Quantification of relative quantity of pEA vectors and dsGFP after digestion. Samples with statistically different amounts of remaining DNA or dsRNA in the cells with respect to the control are indicated by asterisk ($P<0.05$; N=4 (DNA) or N=3 (dsRNA)).

5.3.5 Modulation of RNAi response by BmdsRNase in Hi5 and Bm5 cells

In order to investigate whether BmdsRNase could interfere with RNAi response, a pA-Luc-reporter was transfected into Hi5 cells in combination with different BmdsRNase expression vectors. As it is shown in Fig. 5.4, the presence of 0.1 µg dsRNA-luc could effectively inhibit luciferase reporter activity in Hi5 control cells and the cells co-transfected with expression vectors for BmdsRNase isoforms. Compared with the control cells, only the BmdsRNase(cat) construct showed some minor recovery from RNAi ($P<0.05$) (Fig. 5.5).

Because RNAi efficiency is species and tissues dependent (Terenius et al., 2011) and an obvious RNAi response exists in Bm5 cells (Swevers et al., 2011), it was decided to perform the same experiments in Bm5 cells. Similar results could be observed as in Hi5 cells since, when 0.1 µg dsRNA-luc was co-transfected into Bm5 cells (Fig. 5.5), where only co-expression of BmdsRNase(cat) showed inhibition of RNAi. Although the effect was small, the result was statistically significant ($P<0.05$).

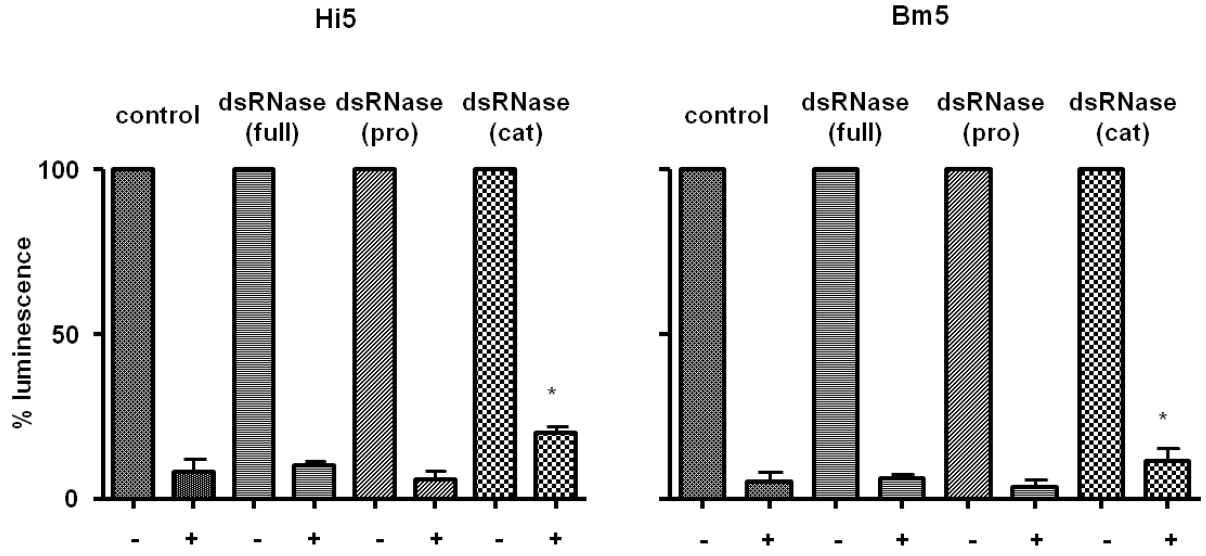


Fig. 5.5. Modulation of RNAi response by BmdsRNases. RNAi response as observed in Hi5 cells and Bm5 cells when luciferase reporter was co-transfected with expression vectors of different BmdsRNase isoforms in the absence (-) or presence (+) of 0.1 µg dsLuc dsRNA. For each condition, luciferase reporter activity in the absence of dsLuc (-) is set at 100%. The second column for each condition shows the luciferase activity in the presence of dsLuc (+) (% activity compared to absence of dsLuc). Samples with significant inhibition of RNAi with respect to the control are indicated by asterisk ($P<0.05$; $N=4$). Only co-expression of BmdsRNase(cat) induced recovery from RNAi.

5.3.6 Immunostaining of BmdsRNase constructs indicates localization in the cytoplasm and co-localization with Flag-tagged Dicer-2

Immunostaining of Hi5 cells for native BmdsRNase (BmdsRNase(full)) detected the

exclusive expression in the cytoplasm, both as diffuse signal and as particles (Fig. 5.6A). Staining for BmdsRNase(pro) showed localization in the cytoplasm of the cells but its distribution was not uniform (Fig. 5.6B). An even more pronounced asymmetric localization in the cytoplasm was also observed for BmdsRNase(cat) where the staining had often a dot-like appearance (Fig. 5.6B). In co-staining experiments, partial overlap was observed with the detection of Flag-tagged Dicer-2 that also showed concentration at specific sites in the cytoplasm (Fig. 5.6B). This type of staining also resembled the asymmetric subcellular localization, which was observed for the RNA binding proteins R2D2 (Swevers et al., 2011). At high magnification, it was also observed that Flag-tagged Dicer-2 appeared as particles in the cellular cytoplasm, while staining of BmdsRNase was more diffuse (Fig. 5.6B). An interesting observation was that, although Dicer-2 protein preferentially accumulates in the cytoplasm, it was sometimes also localized in the nucleus (Fig. 5.6B). It has indeed been reported that Dicer-2, besides its role in cytoplasmic RNAi, can have a nuclear localization where it is implicated in epigenetic regulation and heterochromatin formation (Cernilogar et al., 2011).

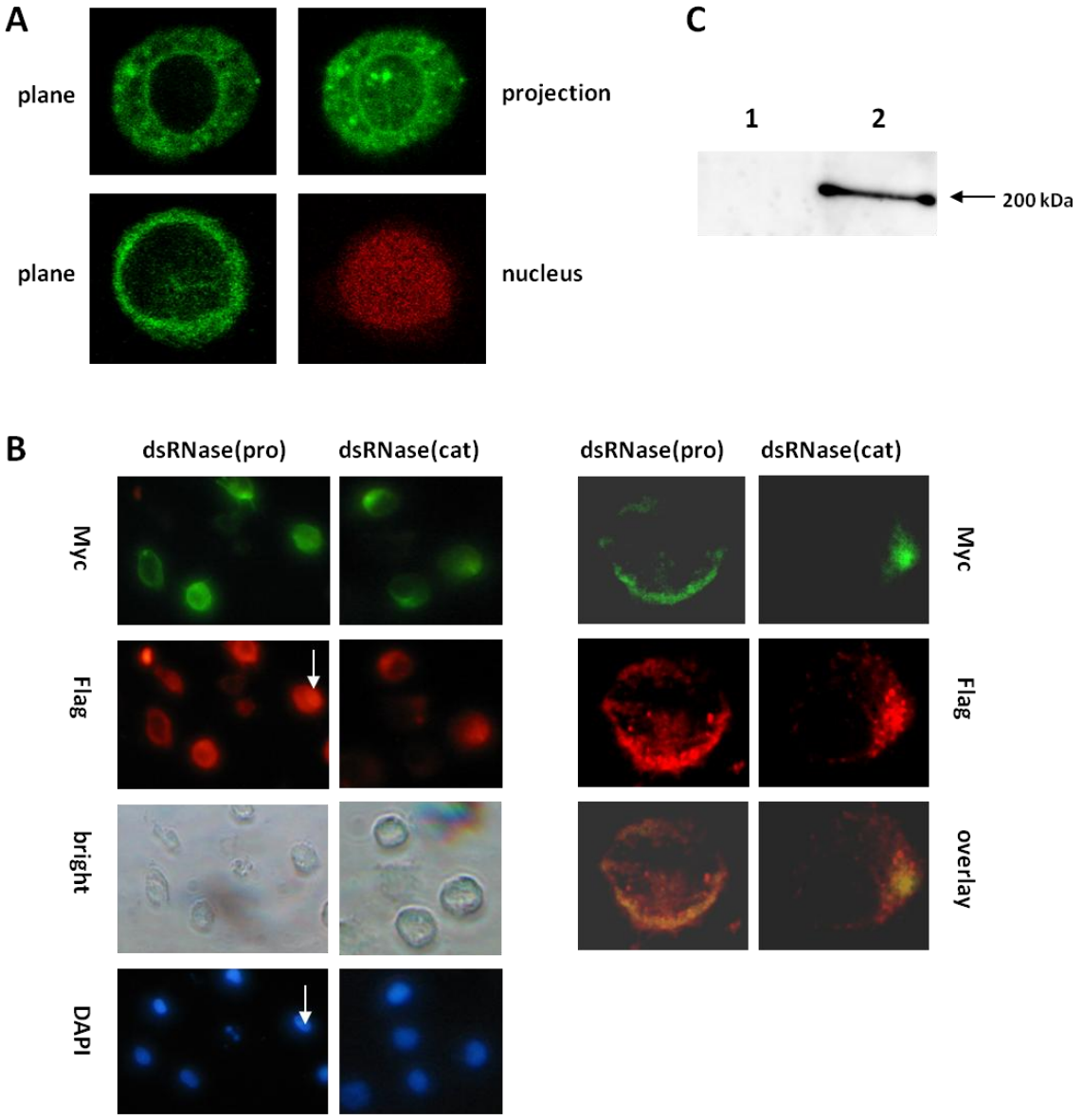
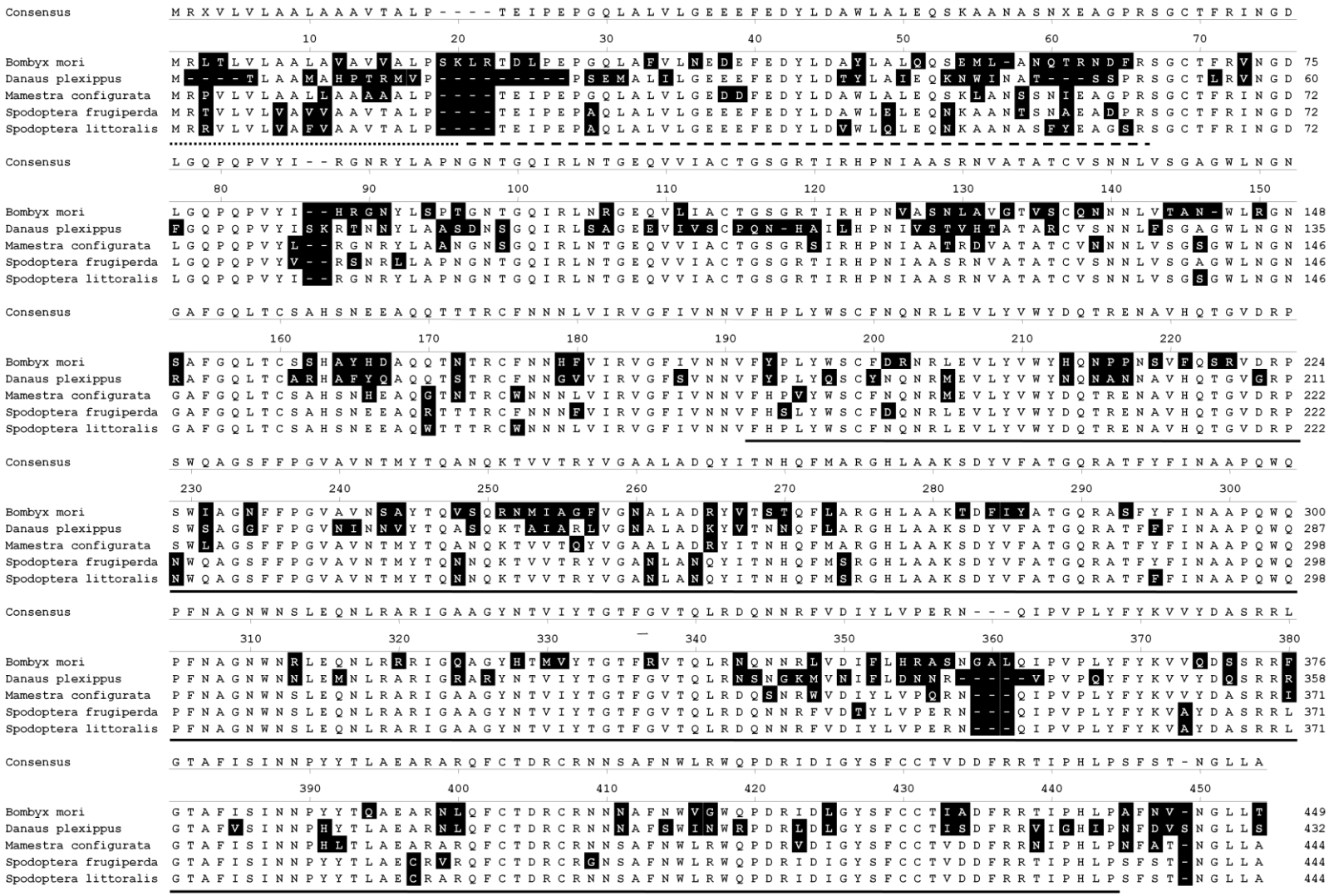


Fig. 5.6. Immunostaining of BmdsRNase in Hi5 cells. (A) Staining of BmdsRNase(full) in Hi5 cells (confocal images; magnification = 200 x). Primary mouse anti-Myc and secondary FITC-labeled anti-mouse antibodies were both used at 1:200. Expression of BmdsRNase(full) was exclusively in the cytoplasm, both as diffuse signal and as particles. Staining of the nucleus in the left lower photograph was carried out using TO-pro-iodide (Molecular Probes; treatment at 1:2000 for 15 min). (B) Co-localization of BmdsRNase(pro) and BmdsRNase(cat) with Flag tagged Dicer-2, which showed asymmetric co-localization in cytoplasm. Primary Flag antibody was used at 1:200, and the secondary AlexaFluor-labeled anti-rabbit antibody was used at 1:500. To the left are shown photographs at low magnification (60 x) taken with the inverted fluorescence microscope while photographs at the right are at high magnification (200 x) and taken with the confocal microscope. The co-localization of nuclear staining of Flag-Dicer-2 and DAPI staining in a single cell is indicated by white arrows in the corresponding photographs. (C) Western blot detection of Flag-Dicer-2 (1: untransfected cells; 2: transfected cells).

5.3.7 Phylogenetic analysis of BmdsRNase

During GenBank searches, homologs of BmdsRNase were found in other lepidopteran insects. Other putative dsRNases with high similarities (79%-85%) but with sequence identities of around 70% were found in *M. configurata, S. frugiperda, S. littoralis* and *D. plexippus*. The protein sequences of these dsRNases were aligned and their DNA/RNA non-specific endonuclease domain is underlined in Fig. 5.7A. When searching the *Bombyx* genomic database, KAIKOBLAST, a paralog of BmdsRNase was found at *Bombyx* Scaffold 40. However, identities between these two *Bombyx* paralogs were 43% (Fig. 5.7B). If an upstream in-frame ATG is taken as the translation start, this paralog has a predicted signal peptide of 17 amino acids (SignalP 4.0 Server, http://www.cbs.dtu.dk/services/SignalP/). However, there is no propeptide predicted by ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/). Sequence alignments between two *B. mori* paralogs also indicated that there was no peptide in the paralog corresponding to the propeptide domain in the BmdsRNase (Fig. 5.7B).

A


Consensus
Bombyx mori
Danaus plexippus
Mamestra configurata
Spodoptera frugiperda
Spodoptera littoralis

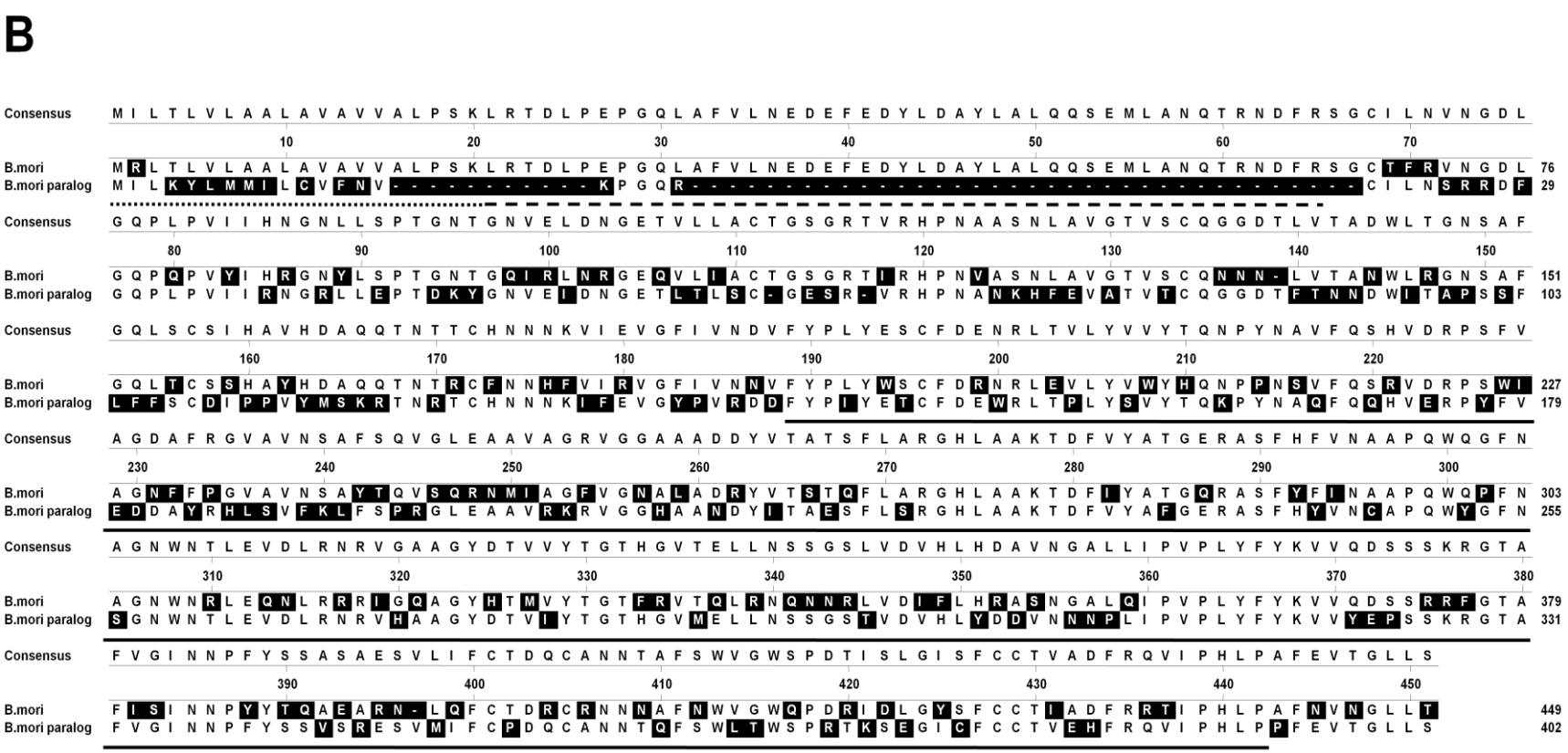


Fig. 5.7. Alignment of the amino-acid sequences of the BmdsRNase homologs and paralog. (A) dsRNases from *B. mori*, *M. configurata*, *S. frugiperda, S. littoralis* and *D. plexippus* and (B) two BmdsRNase paralogs were aligned with ClustalW method. Non-identical amino-acids are boxed in black. Signal peptide domain is underline with round dot and the propeptide domain is underline with dash. DNA/RNA non-specific endonuclease domain is underlined with solid.

Other BmdsRNase homologs found in GenBank only shared 30-40% identities, including alkaline nucleases from *D. plexippus* (a paralog of the enzyme mentioned earlier, see also further below) and *C. quinquefasciatus*. Homologs of nuclease were also obtained in *A. aegypti, G. morsitans, P. camtschaticus, P. monodon, M. japonicus* and *T. castaneum*. Hypothetical or unknown proteins with identities of approximately 30% were found in *A. gambiae, A. darlingi, Drosophila, C. floridanus* and *D. pulex*.

Based on these BmdsRNase homologs and paralog in insects, a phylogenetic tree was constructed (Fig. 5.8). In this tree, BmdsRNase formed a subcluster with other putative dsRNases from *M. configurata, S. frugiperda, S. littoralis* and *D. plexippus*. On the other hand, the BmdsRNase paralog was in another subcluster with a second related protein from *D. plexippus* (55% identities with the *Bombyx* paralog).

The homologs of Diptera also shared the common cluster, which was parallel with the Lepidopteran cluster. This cluster also has two subclusters, one consisting of the homologs from flies and the other from mosquitoes. An ant (Hymenoptera) homolog showed divergence before the appearance of the dipteran and lepidopteran clusters (belonging to the superorder of Mecopterida). However, no homolog was found in the honeybee genome sequence (data not shown). The *Tribolium* (Coleoptera) homolog showed even more divergence and formed an outgroup that was even more distant than crustacean sequences (Fig. 5.8).

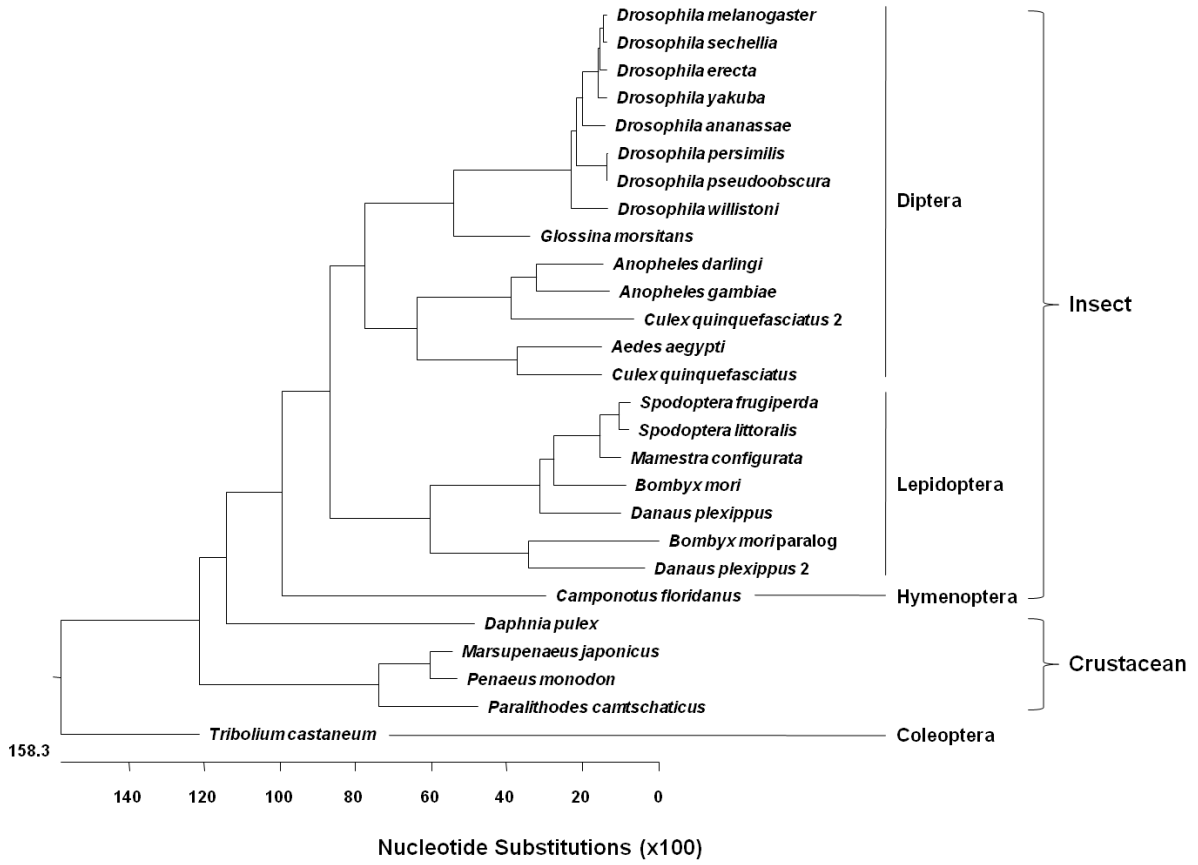


Fig. 5.8. Phylogenetic relationship of BmdsRNase with other homologs and paralog from insect and crustacean species. Phylogenetic tree was constructed based on the BmdsRNase homologs and paralog, including dsRNases of *B. mori*, *M. configurata*, putative dsRNase of *S. frugiperda*, *S. littoralis* and *D. plexippus*, *B.mori* paralog, alkaline nucleases from *D. plexippus* and *C. quinquefasciatus*, nucleases in *A. aegypti*, *G. morsitans*, *P. camtschaticus*, *P. monodon*, *M. japonicus* and *T. castaneum*, hypothetical or unknown proteins in *A. gambiae*, *A. darlingi*, *D. pseudoobscura*, *D. persimilis*, *D. sechellia*, *D. ananassae*, *D. willistoni*, *D. yakuba*, *D. melanogaster*, *D. erecta* , *C. floridanus* and *D. pulex*.

5.3.8 No alternative forms of *BmdsRNase* mRNA detected by 5'-RACE PCR

To determine whether the other two mRNA isoforms of dsRNase (pro and cat) also exist *in vivo* or not, 5'-RACE PCR was performed to detect the possible alternative start sites of the mRNA. According to Fig. 5.9, a fragment of 448 bp was amplified from the midgut tissue. Two fragments (448 bp and 871 bp) were amplified from the epidermis tissue. Sequencing results indicated that the both 448 bp fragments corresponded to *BmdsRNase(full)*; while the 871 bp fragment of epidermis tissue is a PCR artifact since it corresponded to a duplication of sequences of the 448 bp fragment.

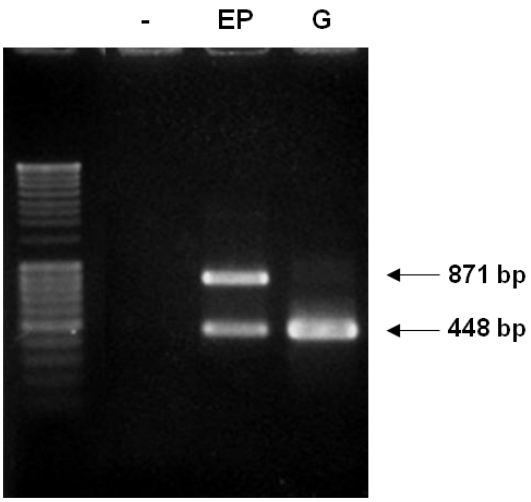


Fig. 5.9. 5'-RACE amplification of *BmdsRNase* from epidermis (EP) and midgut (G).

5.4 Discussion

Our interest in BmdsRNase of *Bombyx* originates from our studies of the mechanism of RNAi in lepidopteran insects. Variation in RNAi efficiency exists in Lepidoptera among different species, tissues, stages and target genes (Terenius et al., 2011). This variation might be due to the uptake and delivery of dsRNA, the intracellular expression levels of RNAi machinery components and/or the presence of RNA degrading enzymes to degrade and clear dsRNA or siRNA.

A unique finding was the broad expression pattern of *BmdsRNase* mRNA in *Bombyx* larval tissues except for the gonads and the hemocytes (Fig. 5.1), although it was originally reported that BmdsRNase was only expressed in the midgut (Arimatsu et al., 2007b). Because of this finding, the functional properties of BmdsRNase, that is a DNA/RNA non-specific nuclease capable to digest DNA and dsRNA (Arimatsu et al., 2007a), were further investigated *in vitro* including its possible functional involvement in the RNAi response. As in the case of *Lygus lineolaris*, saliva containing dsRNase activity was proved to be able to digest dsRNA, which caused negative RNAi response in the dsRNA oral ingestion experiment (Allen and Walker, 2012).

Expression of full-length BmdsRNase in Hi5 cells did not result in its secretion to the extracellular medium (Fig. 5.2B). Substitution of the signal peptide with a chorion signal peptide could also not stimulate the secretion of the protein to the cell growth medium (Fig. 5.3B). Production of secreted proteins by Hi5 cells is usually very efficient as illustrated for the mammalian L1-Fc protein (Fig. 5.3C); (Farrell et al., 2000; Iatrou and Swevers, 2005; Lavdas et al., 2010). Thus, midgut cells in the insect must have a specialized secretion

machinery, that is absent in Hi5 cells, and that allows them to secrete BmdsRNase efficiently. Specialization among different tissues to repress or support secretion has been reported for other proteins: for instance for *Bombyx* arginase, for which two isoforms are known. Interestingly, one isoform is secreted in the extracellular seminal fluid by the male accessory glands in a signal peptide-independent manner, while the other isoform remains intracellular in the other tissues (Nagaoka et al., 2011). Thus, tissue specialization could be a determining factor whether a particular protein is secreted or not. It would be interesting to determine the mechanism by which midgut tissue achieves secretion of BmdsRNase.

The production of BmdsRNase in Hi5 cells allowed us to address the question whether this enzyme could catalyze the degradation of nucleic acids in the cytoplasm of the cells. So far, the functional properties of BmdsRNase have only been investigated *in vitro* using the processed mature protein, corresponding to BmdsRNase(cat) in our experiments, isolated from midgut content (Arimatsu et al., 2007a; Arimatsu et al., 2007b). Investigation of the intracellular function of BmdsRNase is relevant because homologs of BmdsRNase have been reported to function in intracellular nucleic acid metabolism (Cote and Ruiz-Carrillo, 1993; Nestle and Roberts, 1969; Robertson et al., 1968) and BmdsRNase has been hypothesized to function in the innate immune response against *Bombyx* cytoplasmic polyhedrosis virus (CPV), a virus with dsRNA genome structure (Wu et al., 2009; Mori and Metcalf, 2010). Such defense function would require intracellular expression of the enzyme where it could attack exposed replication intermediates efficiently. The RNAi machinery, consisting of Argonaute 2, Dicer-2 and R2D2, has also been implicated in antiviral defense (Saleh et al., 2009) and dsRNase activity could contribute to the further degradation of cleaved products from the RISC complexes.

Judged from the relative MWs of the expressed proteins, correct processing of BmdsRNase(full) to BmdsRNase (pro) or BmdsRNase(cat), which occurs in midgut tissue, wass not observed in Western blot analysis (Fig. 5.2B). But it remains unclear whether the alternative forms of *BmdsRNase* mRNA exist in the larval tissues that encode BmdsRNase(pro) and BmdsRNase(cat) (without proteolytic processing). It is possible that alternative exons exist in the *BmdsRNase* gene that may be spliced to common exons and provide alternative translation start sites. Thus, a 5'-RACE PCR was performed in the larval midgut and epidermis tissues (Fig. 5.9). However, sequencing of amplified fragments showed there was no alternative splicing of the mRNA at the 5'-end. Only evidence of the full form of *BmdsRNase* mRNA was obtained in the larval tissues. This could indicate that this type of processing is unique in the midgut tissue. Thus it will require the generation of specific

antibodies to check the possible expression of processed protein isoforms in non-midgut tissues of *Bombyx* to validate this. For comparative purposes, and to determine whether processing of BmdsRNas(full) is necessary for functional activity, expression constructs corresponding to the intermediate steps in the maturation process in the midgut were also generated and tested for expression and in functional assays in Hi5 cells.

Our experiments indicated that the isoform BmdsRNase(cat), corresponding to the mature peptide present in the digestive juice, also exhibited the highest activity when expressed as an intracellular protein. This could be observed in assays that monitored degradation of DNA and dsRNA (Fig. 5.4) and dsRNA-mediated gene silencing (Fig. 5.5), where inhibition of the RNAi response was observed, presumably through degradation of transfected long dsRNAs or processed siRNAs. Immunofluorescence staining of transfected Hi5 cells also showed partial co-localization of BmdsRNase(cat) with Flag-tagged Dicer-2 at asymmetric localizations in the cytoplasm (Fig. 5.6B). BmdsRNase(pro) possessed similar partial co-localization as well, although its functionality was not found. On the other hand, clear intracellular dsRNase activity was also observed after expression of BmdsRNase(full), albeit at lower levels than BmdsRNase(cat). This finding indicates that full processing to the mature form in the cytoplasm of the cells is not necessary for functional activity and points to the relevance of expression of BmdsRNase(full) in larval non-midgut tissues *in vivo*. The activation of BmdsRNase(full) might play a role for this enzyme in the antiviral innate immune response, which was observed following CPV infection (Wu et al., 2009). This could also explain the high efficiency in nucleic acids cleavage but small rate in RNAi response (Fig. 5.5).

Experiments that employ the active enzyme purified from the digestive juice established that BmdsRNase only works well under alkaline conditions (Arimatsu et al., 2007a). However, detectable activity of BmdsRNase can clearly be observed when expressed intracellularly (Fig. 5.4), while the pH is slightly alkaline (around 7.2) in the cytosol of most resting cells. Our results indicate that all the properties of the enzyme are not fully understood and that it could behave differently intracellularly than as a secreted form.

In summary, while the role of dsRNase in the digestion of nucleic acids in the food is well established, this study had the ambition to investigate other functions of this enzyme in intracellular nucleic acids digestion as well as the intracellular RNAi response. Our study illustrated the broad expression pattern of *BmdsRNase* mRNA in different larval tissues besides the midgut. Our approach using tissue culture cells and engineered constructs necessarily is artificial and only partial answers are obtained. Expression of different forms of

BmdsRNase could only be detected in the pellets and supernatants of Hi5 cell extracts. The absence of secretion of the full-length BmdsRNase by Hi5 cells points to a specialized machinery of secretion in differentiated midgut cells. However, intracellular functions for the enzyme were uncovered in this work. BmdsRNase(cat) can degrade both DNA and dsRNA, of which the latter property results in a small effect on the RNAi response in both Hi5 and Bm5 cells. Of special interest is the capacity of BmdsRNase(full) to degrade dsRNA intracellularly, with possible relevance for the innate immune response against invading nucleic acids with dsRNA structure.

Chapter 6

Transcriptional response of BmToll9-1 and RNAi machinery genes to exogenous dsRNA in the midgut of *Bombyx mori*[*]

[*] Adapted from:

Jisheng Liu, Guy Smagghe, Luc Swevers (2013). Transcriptional response of BmToll9-1 and RNAi machinery genes to exogenous dsRNA in the midgut of *Bombyx mori*. Journal of Insect Physiology 59: 646-654.

6.1 Introduction

RNAi is an evolutionary conserved defense response against virus infection or dsRNA structure molecules, but virus or dsRNA could also trigger other pathways in the immune response besides RNAi (Merkling and van Rij, 2012).

The innate immune system is the first and only host defense line in insects due to the lack of the adaptive immune system (Cheng et al., 2008). In this process, Toll receptors play a key role in the innate immunity (Takeda and Akira, 2005). However, it remains unknown whether dsRNA could also be recognized as 'foreign' and interact with immunity-related Toll receptors in insects. There are 14 Toll-related genes identified in the silkmoth *B. mori*, (Tanaka et al., 2008).

In mammalian genomes, at least 11 Toll-like receptors (TLRs) have been identified (Takeda and Akira, 2005). These mammalian TLRs are typical pattern recognition receptors (PRRs) which are able to recognize their specific pathogen-associated molecular patterns (PAMPs) in the immune responses. In contrast to insect Toll receptors, different pathogen-associated ligands could be identified for mammalian TLRs (Takeda and Akira, 2004). For instance TLR3 is an endosome receptor for dsRNA (Alexopoulou et al., 2001) and it has been reported that it can interfere with siRNA mediating gene suppression (Kariko et al., 2004).

In this chapter, the expression profiles of the 14 Toll-related genes were determined in silkmoth 5th instar larvae and pupae as well as the silkmoth-derived Bm5 cell line. Different ways of dsRNA delivery were applied in the *Bombyx* larvae, studying the possible effects of dsRNA on *BmToll9-1* expression. Because lipopolysaccharide (LPS) was proven to affect expression of other Toll receptors, such as *BmToll7-2* (Imamura and Yamakawa, 2002), application of LPS was also performed. The persistence of dsGFP was tested in hemolymph and midgut extracts to explain the absence of effects of dsRNA on *BmToll9-1* expression by feeding in the larvae. The expression of two RNAi machinery genes, *BmDcr2* and *BmAgo2*, was also tested after dsRNA treatment.

6.2 Material and methods

6.2.1 Experimental animals

The larvae of *B. mori*, Daizo strain, were reared on artificial diet (Yakuruto Co., Japan) at 25°C under a photoperiod of 12 h light and 12 h dark. Larval tissues were dissected from 5th instar larvae at day 4-5 after the molt. Tissues from 3-10 larvae were collected in eppendorf tubes on ice and samples were frozen at -70ºC until further processing for RNA extraction. To isolate hemocytes, hemolymph was collected on ice after cutting the first

proleg and subsequently centrifuged at low speed (800 g) at 4ºC to collect the cell pellet. The following tissues were used in the subsequent experiments: epidermis, fat body, midgut, thoracic muscles, Malpighian tubules, brain, silk glands, testis, ovaries and hemocytes.

6.2.2 RNA extraction and RT-PCR

Frozen tissues were homogenized in TRI Reagent (Sigma) and total RNA was extracted according to the manufacturer's protocol. The quantity of extracted RNA was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and/or by electrophoresis on 1% (w/v) agarose gels. First-strand complementary DNA (cDNA) from 1 µg RNA was synthesized using a SuperScript II reverse transcriptase (Invitrogen) and a Oligo(dT)$_{12-18}$ primer (Invitrogen). Detailed RNA extraction was described by Machado et al. (2007).

PCR optimization was performed with different cycle numbers and gradient temperature. Primers initially used for detection of mRNA of *BmToll* receptors by PCR are listed in Table 6.1. Template cDNA was denatured at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 40 s for each cycle. Control PCRs employed primers to amplify *Actin* mRNA (Machado et al., 2007). A final elongation step was used at 72°C for 10 min. PCR products were run on 1.5% agarose gels and stained with ethidium bromide.

Table 6.1. Primer sets used for mRNA amplification of *BmToll* receptors

Genes	Primer Pair	Product Size
BmToll3-1	CGAGCACCTCTTGCGACTTGACC (forward)	457 bp
	CGATAACCATCTACGGGGGGGAG (reverse)	
BmToll3-2	GCAGTGATGGTCGCAGTTAC (forward)	483 bp
	GTTCCAGAGCAGGTAGGTGTT (reverse)	
BmToll3-3	CGTGGCTGCATCACTCTGTTT (forward)	466 bp
	GTCGATCCTGGCGTCGTTAGT (reverse)	
BmToll6	CGTTACTGTTGATAGCCCTGTG (forward)	541 bp
	AACTGTCTATCACCCCATTCG (reverse)	
BmToll7-1	ACAACCTTCCGTTATTGGCGT (forward)	536 bp
	CCGTTTCTCCTTCCATGTTATCTC (reverse)	
BmToll7-2	TTCCCATGATGGTTTCAACTC (forward)	572 bp
	TATATACCTGAGACGCTCCCAG (reverse)	

BmToll7-3	TGCCTATGATGGTAACGACTC (forward)	515 bp
	TTTCAAGTACGGCCTTAAATC (reverse)	
BmToll8	CATACCTCTTCTTATCGCAACAC (forward)	554 bp
	GTGCAAACTTCAACTTCTCCC (reverse)	
BmToll9-1	TTGTGCGTCGTTTGCTTCGG (forward)	375 bp
	TGGAGGCAGACGCTGATGTT (reverse)	
BmToll9-2	CGTTGCGATGCCTGATG (forward)	430 bp
	CACCATTGGGATTTAGCA (reverse)	
BmToll10-1	CGCCTTCGGACTTTCTTTGC (forward)	672 bp
	ATGAAGCCCGTATCTTTGGT (reverse)	
BmToll10-2	TCAGCGTTTACTTTGCCTCA (forward)	331 bp
	GAAAGCAAGAACAGCCCTCA (reverse)	
BmToll10-3	CTGTAGATGGAAACAACTGG (forward)	556 bp
	GCACTCACTAAACTATCACCC (reverse)	
BmToll12	GACGACAGCCTACAGCAA (forward)	308 bp
	GGTGAGTCCCTAAGTAACAG (reverse)	

To detect the expression of *BmdsRNase* (primers listed in Table 6.2), template cDNA was denatured at 94°C for 2 min, followed by 24 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for each cycle. To check the expression of *BmDcr2* and *BmAgo2* (primers information in Table 6.3), template cDNA was denatured at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s for each cycle or 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 25 s for each cycle. A final elongation step was used at 72°C for 10 min. PCR products were run on 1.5% agarose gels and stained with ethidium bromide.

Table 6.2. Primer set used for mRNA detection of *BmdsRNase*

Genes	Primer Pair	Product Size
BmdsRNase	GATCCGTCACCCCAATGTTG (forward)	421 bp
	GCATTACCCACGAATCCAGC (reverse)	

Table 6.3. Primer sets used for mRNA detection of *BmDcr2* and *BmAgo2*

Genes	Primer Pair	Product Size
BmDcr2	CATACAGTTCACCGAAGAGG (forward)	449 bp
	GGATGTACGACGAGTGAGAC (reverse)	

BmAgo2	TCTCCGATTGACTTGGGCGAC (forward) ATACGGTCATCCTAACCGGCG (reverse)	370 bp

To detect the expresion of dsGFP in bacteria, total RNA from bacteria was extracted as described above. First strand cDNA from 1 µg RNA was synthesized with SuperScript II reverse transcriptase and dsGFP-specific primer (5'-CTACCCCGACCACATGAAGC-3'). PCR was performed with the forward primers (listed in Table 6.4), with an amplification program of 94°C 30 s, 55°C 30 s, 72°C 25 s, for 30 cycles.

Table 6.4. Primer set used to detect the expression of dsGFP in bacteria

Genes	Primer Pair	Product Size
dsGFP	GCTTCTCGTTGGGGTCTTTG (forward) TCCAGGAGCGCACCATCTTC (reverse)	421 bp

6.2.3 dsRNA synthesis

pGEM-T vector (Promega) containing a GFP fragment was linearized by NcoI (Fermentas). The T7 promoter was added to the GFP fragment by PCR with the forward primer 5'- TAATACGACTCACTATAGGGTACGGCGTGCAGTGCT and reverse primer 5'- TAATACGACTCACTATAGGGTGATCGCGCTTCTCG (T7 promoter sequence is underlined). PCR conditions were 94°C for 2 min, followed by 5 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 70 s, and another 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 70 s. A final elongation step was used at 72°C for 3 min. dsGFP synthesis using the PCR fragment as template was carried out with MEGAscript RNAi kit (Applied Biosystems).

6.2.4 dsRNA feeding assay in the *Bombyx* larvae

Freshly molted 5th instar larvae were individually placed into each well of a 24-well plate and starved for 24 h. Each larva was provided the same amount of artificial diet coated with 50 µg of LPS (Sigma) or dsGFP in 100 µl MQ; diet coated with 100 µl of MQ was used as the negative control. Treated larvae were collected at 0, 3, 6 and 24 h after initiation of feeding. Midgut, epidermis and fat body were dissected and sampled in TRI Reagent. RT-PCR to detect the expression of BmToll9-1 was performed with the same primers and conditions as mentioned above. Quantification of PCR fragments in agarose gel was carried out by Quantity One 4.6.2 software (Bio-Rad). Quantities of PCR fragments corresponding to the housekeeping gene *Actin* were used for normalization. Statistical analysis of differences

in normalized expression levels was carried out by one-way ANOVA analysis.

6.2.5 dsRNA injection to the *Bombyx* larvae

Freshly molted 5th instar larvae, 24 h after molting, were used for injection experiments. Larvae were anaesthetized and immobilized by submersion into water for 10-15 min. Their surface was sterilized with 70% ethanol and immobilized larvae were injected laterally with a 10 µl-Microliter™ Syringe (Hamilton) between the 2nd and 3rd abdominal segment. Each larva was injected with 5 µg of LPS in 10 µl MQ or 5 µg of dsGFP in 10 µl MQ. For the negative control, each larva was injected with 10 µl of MQ. Samples were collected at 0, 3, 6 and 24 h after injection and subjected to RNA extraction and RT-PCR as described above. Detection of the expression of the housekeeping gene *Actin* was used for normalization to quantify the differences in BmToll9-1 expression. Quantification of BmToll9-1 PCR fragments was carried out by Quantity One 4.6.2 software and statistical differences were analyzed by one-way ANOVA.

6.2.6 dsRNA *in vitro* degradation assay

Freshly molted 5th instar larvae, which were starved for 24 h, were used to isolate the midgut juice. Midgut juice was collected from the midgut by high speed centrifugation (20,000 g) at 4ºC for 10 min as described by Ayra-Pardo et al. (2007). Hemolymph was collected by squeezing the 5th instar larvae body after cutting the first proleg and a grain of phenylthiocarbamide (Sigma) was added to inhibit the melanization. Two micrograms of dsGFP were mixed with midgut juice or hemolymph to a final volume of 30 µl and incubated at 25°C. dsGFP was incubated in DNase/RNase free water as a negative control. After incubation, dsRNA was recovered by RNeasy Mini Kit (Qiagen). dsRNA bound to the column was eluted by 30 µl of DNase/RNase free water and 5 µl of dsRNA solution was run on 1.5% agarose gels and stained with ethidium bromide. A MassRuler DNA ladder mix (Thermo Scientific) was used and 2 µl of the ladder mix loaded to the gel.

6.2.7 Bacteria feeding assay in the *Bombyx* larvae

To generate bacteria that produce dsGFP, a HindIII-XhoI fragment that contains the complete ORF of GFP was cloned into the vector pLitmus28i and transformed into *Escherichia coli* HT115 (DE3), an RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity. Because of the presence of two T7 RNA polymerase promoters flanking the insert in the pLitmus28i vector, RNA of both polarities is produced at high levels in the

induced bacteria. A single colony of *E. coli* was grown in LB medium containing proper antibiotics, at 37°C and 200 rpm overnight. Then the solution of bacteria was diluted 100 fold with LB medium and allowed to grow to OD600 = 0.4. IPTG was added to a final concentration of 1 mM to induce the expression of dsRNA. The culture was incubated at 37°C and 200 rpm for 5 h. Bacterial cells were collected at 10,000 g for 5 min and resuspended in DNase/RNase free water at the ratio of 5:1 (concentrating the bacteria and removing the antibiotics). Bacteria without the pLitmus28i vector containing the GFP insert were used as control for absence of dsRNA production.

Freshly molted 5th instar larvae were individually placed into each well of a 24-well plate and starved for 24 h. Each larva was provided the same amount of artificial diet coated with 40 µl of bacteria solution. Samples were collected at 0, 3, 6 and 24 h after feeding of bacteria. RT-PCR and quantification of BmToll9-1 expression were carried out as described above.

6.2.8 Phylogenetic analysis

The phylogenetic tree was constructed using MEGA4 by performing Neighbor-joining method using ClustalW alignment (Tamura et al., 2007). The sequence information of BmTolls was acquired from Tanaka et al. (2008). HsTLR3 (ABC86910), HsTLR4 (AAY82270), DmToll9 (NP_649214) and AgToll9 (AF444782_1) were from NCBI database. The values obtained in the bootstrap test are indicated.

6.3 Results

6.3.1 Broad expression pattern of *Bombyx* Toll receptors in larvae and pupae

As shown in Fig. 6.1A, all the Toll receptors were widely expressed in all the tissues in the 5th larval instar. The expression patterns of *BmToll3-1* and *3-2* were similar with strong expression levels in the midgut, Malpighian tubules, testis, ovaries and hemocytes. The expression levels of *BmToll3-1, 3-2* and *3-3* were relatively low in the epidermis and fat body. *BmToll7-1* and *7-3* had similar expression profiles, with relatively high expression in the reproductive glands which was also observed for *BmToll6*. But in epidermis, fat body, Malpighian tubules and silk glands, both *BmToll7-1* and *7-3* had lower expression. For *BmToll8*, expression was observed in each tissue but with relative low levels in epidermis and fat body. The expression pattern for the three *BmToll10* receptors was similarly broad though lower expression levels in the silk glands and hemocytes for *BmToll10-2* and brain and silk glands for *BmToll10-3* were observed. As for *BmToll12*, similar expression levels were

present in all the tissues. To our interest, for the BmToll9 genes, *BmToll9-2* had enriched expression in Malpighian tubules and brain. Interestingly, *BmToll9*-1 had abundant expression in the midgut and relative high expression in muscles and Malpighian tubules.

When the expression profiles in the pupal tissues and the Bm5 cell line were determined, *BmToll9-1* also had highest expression in the midgut but very weak to absent expression in Bm5 cells (Fig. 6.1B). At the pupal stage, *BmToll3-1, 7-1, 7-2, 7-3, 8, 10-2, 10-3* were expressed in every tissue as well as the Bm5 cells. *BmToll3-2* expression was absent in the wing discs. *BmToll6* and *10-1* showed a relative enrichment in the midgut, Malpighian tubules, brain and Bm5 cells; *BmToll10-1* also had relatively high expression in the reproductive glands. *BmToll12* was strongly expressed in brain and testis, while it was poorly expressed in the wing discs. In general, Toll genes had broad expression pattern in both larval and pupal tissues, as well as in Bm5 cells.

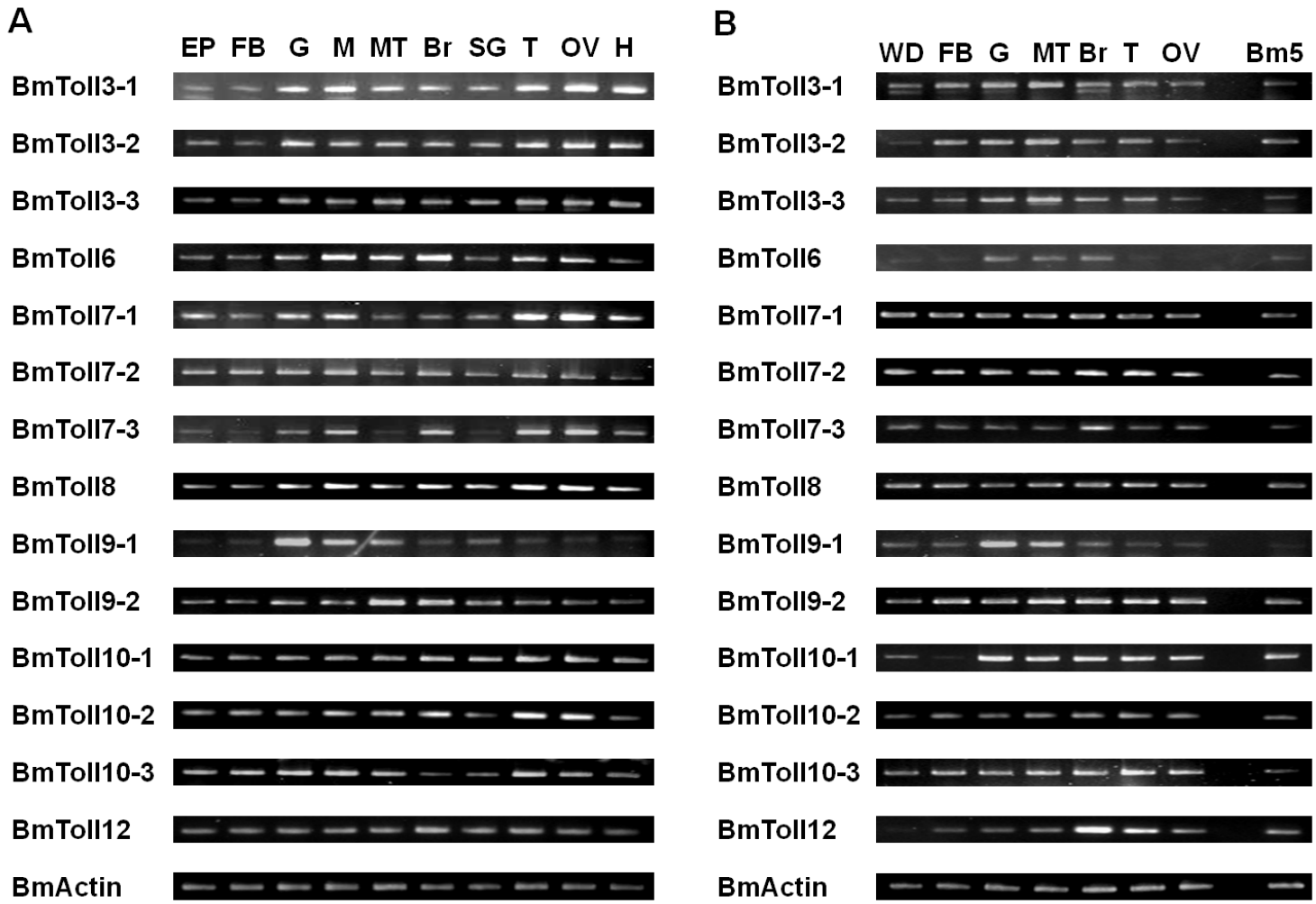


Fig. 6.1. *In vivo* expression pattern study of *BmToll* mRNAs in different (A) larval tissues, (B) pupal tissues and Bm5 cells. Fragments were obtained after 35 cycles of PCR. Larval tissues, epidermis (EP), fat body (FB), midgut (G), muscle (M), Malpighian tubules (MT), brain (Br), silk gland (SG), testis (T), ovaries (OV) and hemocytes (H), were collected from 5th instar larvae at day 4-5 after the molt. Pupal tissues, wing discs (WD), fat body (FB), midgut (G), muscle (M), Malpighian tubules (MT), brain (Br), testis (T) and ovaries (OV) were collected from 5th instar larvae at day 5-7 after the pupation. Bm5 cells (Bm5) were also collected at day 4-5 after subculturing.

6.3.2 Phylogenetic analysis of Toll receptors

Phylogenetic analysis indicated that BmToll9-1 and BmToll9-2 are relatively closely related to HsTLR3 among the 14 *Bombyx* Toll-related genes (Fig. 6.2A), raising the question whether these Toll9 receptors could also interact with dsRNA. Because of the dominant expression of BmToll9-1 in the midgut (Fig. 6.1) and the more close evolutionary relationship between BmToll9-1 and HsTLR3 than other *Bombyx* Toll receptors (Fig. 6.2B), we decided to focus on BmToll9-1.

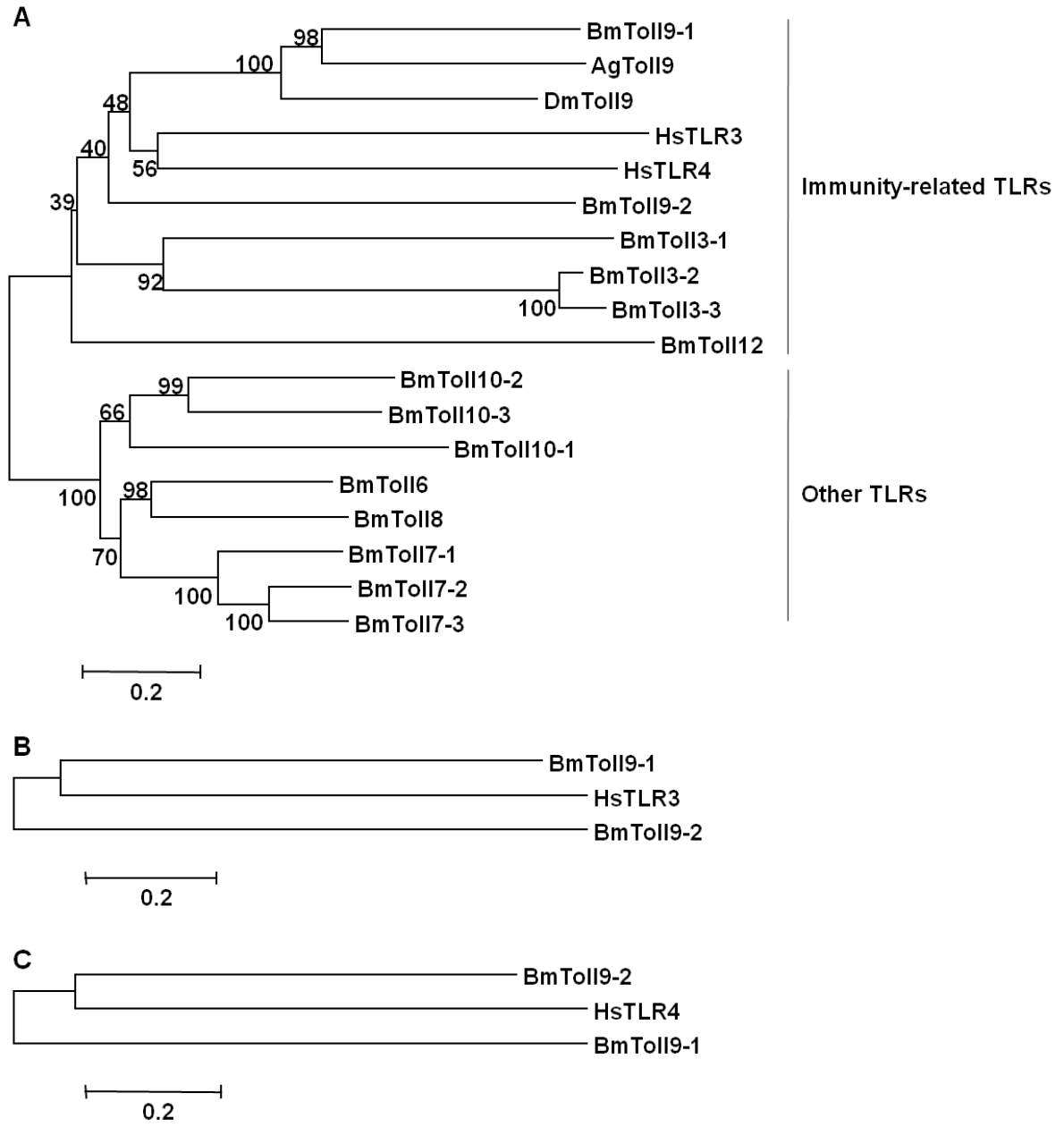


Fig. 6.2. Phylogenetic analysis of BmToll receptors with HsTLR3, DmToll9 and AgToll9. The phylogenetic tree was constructed using MEGA4 by performing Neighbor-joining method using ClustalW alignment (Tamura et al., 2007). The sequence information of BmTolls was acquired from Tanaka et al. (2008). HsTLR3 (ABC86910), HsTLR4 (AAY82270), DmToll9 (NP_649214) and AgToll9 (AF444782_1) were from NCBI database. The values obtained in the bootstrap test are indicated.

6.3.3 Feeding of dsRNA does not affect the expression of *BmToll9-1* mRNA in the *Bombyx* larvae

To examine whether introduction of dsRNA to the *Bombyx* larvae could affect expression of *BmToll9-1* transcription, several different ways of dsRNA delivery were tested, including feeding of dsRNA and injection of dsRNA. Because of its reported effects on expression of other Toll receptors (Imamura and Yamakawa, 2002), LPS was also tested.

As shown in Fig. 6.3A-B, feeding of dsGFP during 3 h could slightly suppress the expression of *BmToll9-1* mRNA in the midgut, but this effect was not significantly different. On the contrary, LPS could clearly suppress the expression of *BmToll9-1* mRNA at 3 h after feeding (Fig. 6.3C-D). Feeding with MQ water had no effect on the expression of *BmToll9-1*, which was constantly expressed in the midgut (Fig. 6.3E-F). No expression was detected in the epidermis and fat body, even though the fat body is the immune organ in the insects (data not shown).

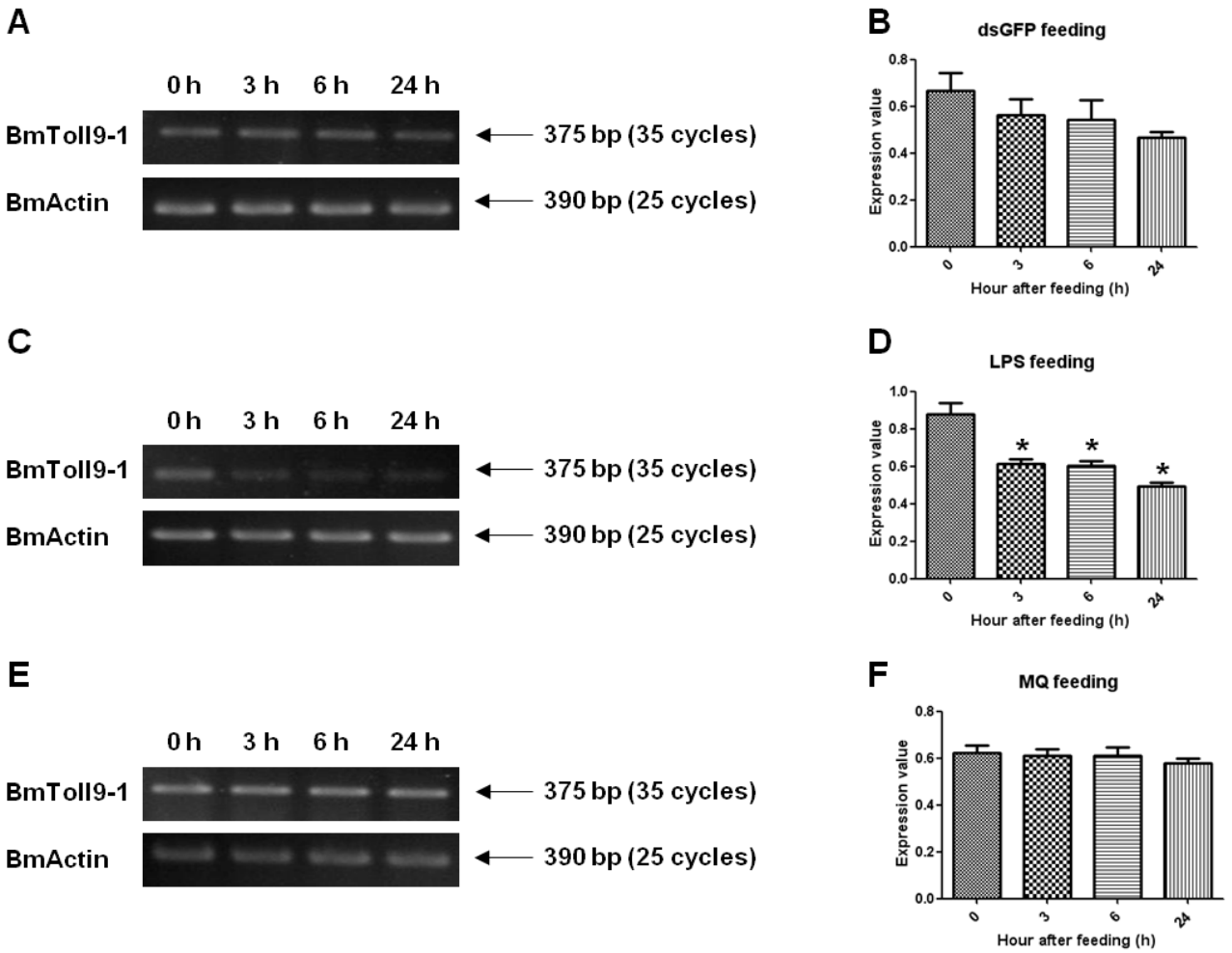


Fig. 6.3. Expression of *BmToll9-1* after feeding assay. Detection of *BmToll9-1* mRNA after feeding with 50 µg of (A) dsGFP, (C) LPS in (E) 100 µl of MQ water. Total RNA was extracted after feeding at 0, 3, 6 and 24 h. One microgram of RNA was used to perform RT-PCR to detect the expression of *BmToll9-1*. Quantification of relative expression quantity of *BmToll9-1* after feeding with (B) dsGFP or (D) LPS or (F) MQ water. Samples with statistically difference with respect to the control (0 h) are indicated by asterisk ($P<0.05$, $N=3$).

6.3.4 Injection of dsRNA suppresses expression of *BmToll9-1* mRNA in the larval midgut

Because feeding of dsRNA did not influence the expression of *BmToll9-1* mRNA in the larvae, a possible explanation could be the presence of dsRNA-degrading enzymes (such as dsRNase) in the midgut content that degrade dsRNA (Liu et al., 2012). Thus, injection of dsRNA was also tested.

In the injection test, clear reduction in the expression of *BmToll9-1* mRNA in the midgut was observed at 3 h after injection of dsGFP. There was no expression detected after 6 and 24 h (Fig. 6.4A-B). Injection of LPS resulted in instant and long term effects, in which the expression of *BmToll9-1* was entirely suppressed starting from 3h (Fig. 6.4C-D). Injection of MQ water did not affect the expression of *BmToll9-1* mRNA and constant expression was detected (Fig. 6.4E-F).

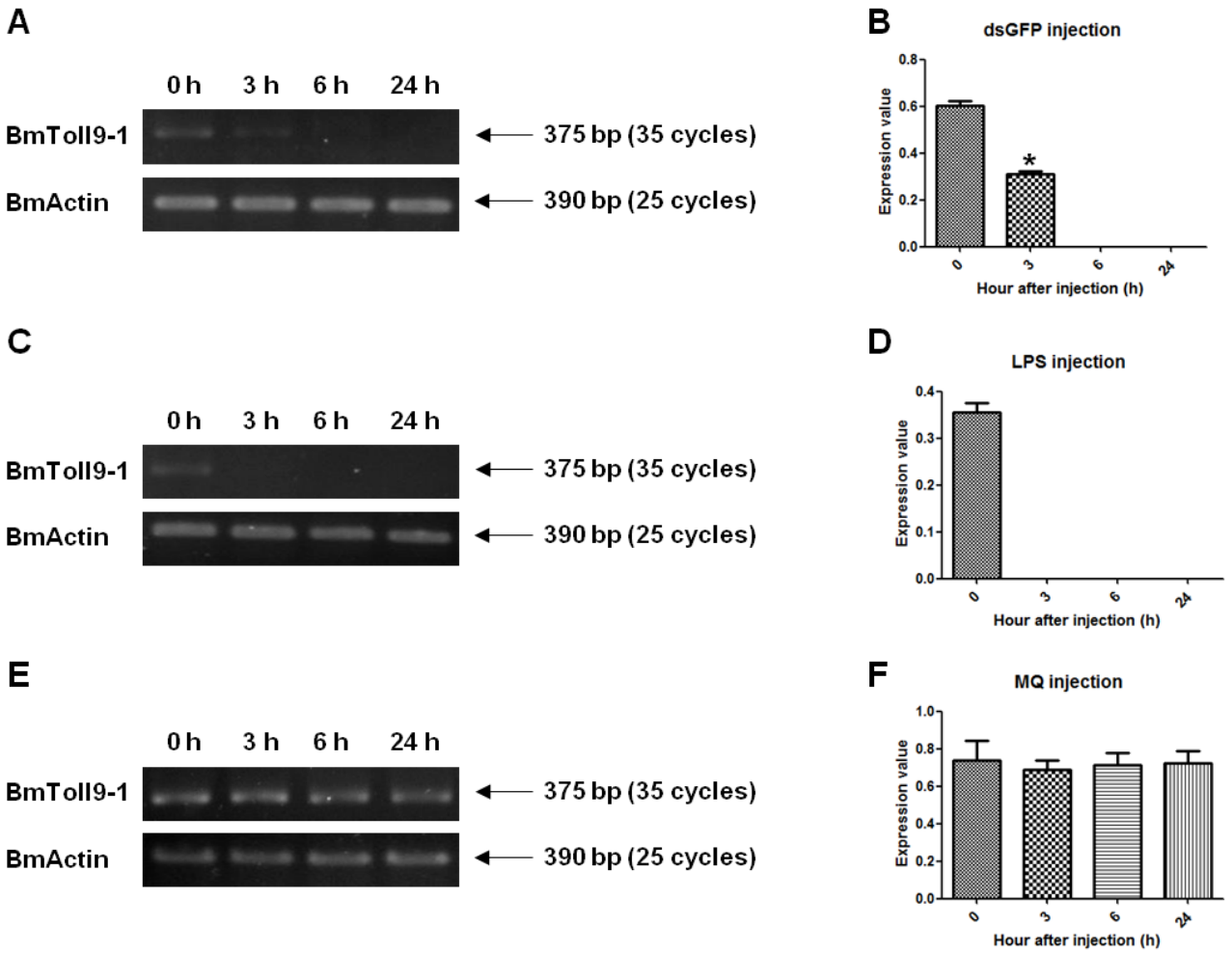


Fig. 6.4. Expression of *BmToll9-1* after injection assay. Detection of *BmToll9-1* mRNA after injection with 5 µg of (A) dsGFP, (C) LPS in (E) 10 µl of MQ. Total RNA was extracted after feeding at 0, 3, 6 and 24 h. One microgram of RNA was used to perform RT-PCR to detect the expression of *BmToll9-1*. Quantification of relative expression quantity of *BmToll9-1* after injection with (B) dsGFP or (D) LPS or (F) MQ water. Samples with statistically difference with respect to the control (0 h) are indicated by asterisk ($P<0.05$, $N=3$).

6.3.5 Higher persistence of dsRNA integrity in the hemolymph than in the midgut

In order to investigate the persistence of dsRNA in the midgut and hemolymph, dsGFP was incubated in the midgut juice and hemolymph *in vitro* for up to 24 h.

The incubation assay indicated that dsGFP was stable in the DNase/RNase-free water for up to 24 h. On the contrary, midgut juice could degrade dsGFP in a very short period. After incubation for 10 min, dsGFP showed smearing on the gel, indicating that degradation had already started. There was no signal detected after 30 min, which suggested that dsGFP had been entirely degraded already (Fig. 6.5A). On the other hand, dsGFP degradation occurred at a much lower rate in the hemolymph than in the midgut. Intact dsGFP was still present in the hemolymph after incubation for 3 h, even though the band started to smear. After 6 h, the corresponding band of dsGFP was not detected and only some smearing in the small molecular weight range was observed. After 24 h, no signal was detected on the gel (Fig. 6.5B).

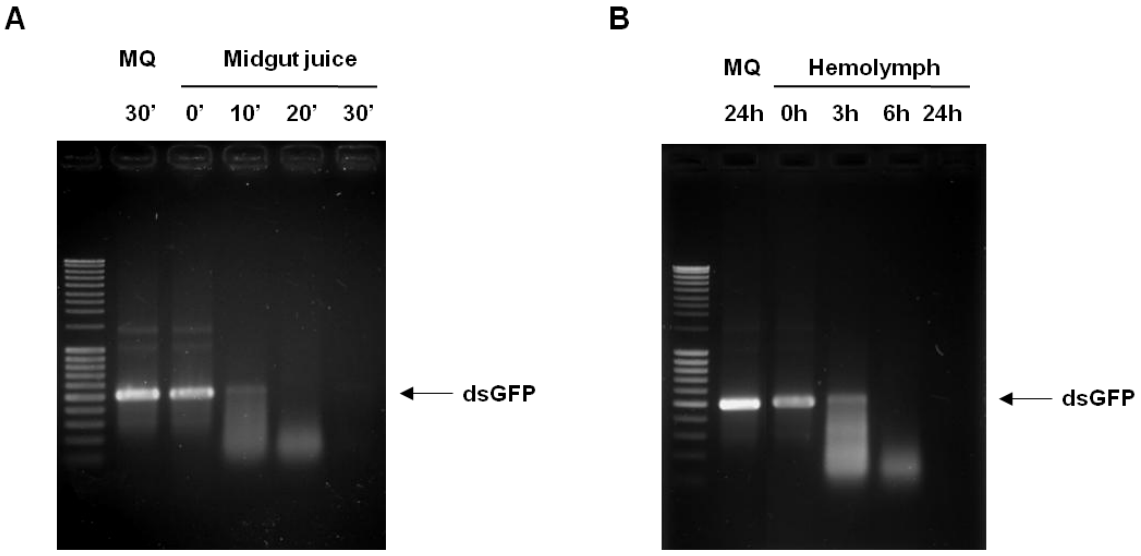


Fig. 6.5. Persistence of dsRNA incubated in midgut juice and hemolymph. Two micrograms of dsGFP were mixed with (A) midgut juice or (B) hemolymph to the volume of 30 µl and incubated at 25°C. dsGFP was incubated in DNase/RNase-free water as a negative control. Two microliters of the ladder mix was loaded to the gel.

6.3.6 Activation of *BmdsRNase* by dsRNA injection but not by dsRNA feeding

As shown in Fig. 6.6A-B, feeding of dsGFP didn't alter the expression of *BmdsRNase*, which might be due to the rapid degradation of dsGFP in the midgut content. Similar results were achieved by introduction of water by feeding or injection, where the expression of *BmdsRNase* was stable (Fig. 6.6C-D, G-H). On the other hand, the expression of *BmdsRNase* was significantly increased after 3 h of dsGFP injection (Fig. 6.6E). These significant

differences were also present at 6 h after injection (Fig. 6.6F), which means that the presence of dsRNA in the hemolymph can trigger the expression of *BmdsRNase* mRNA in the midgut.

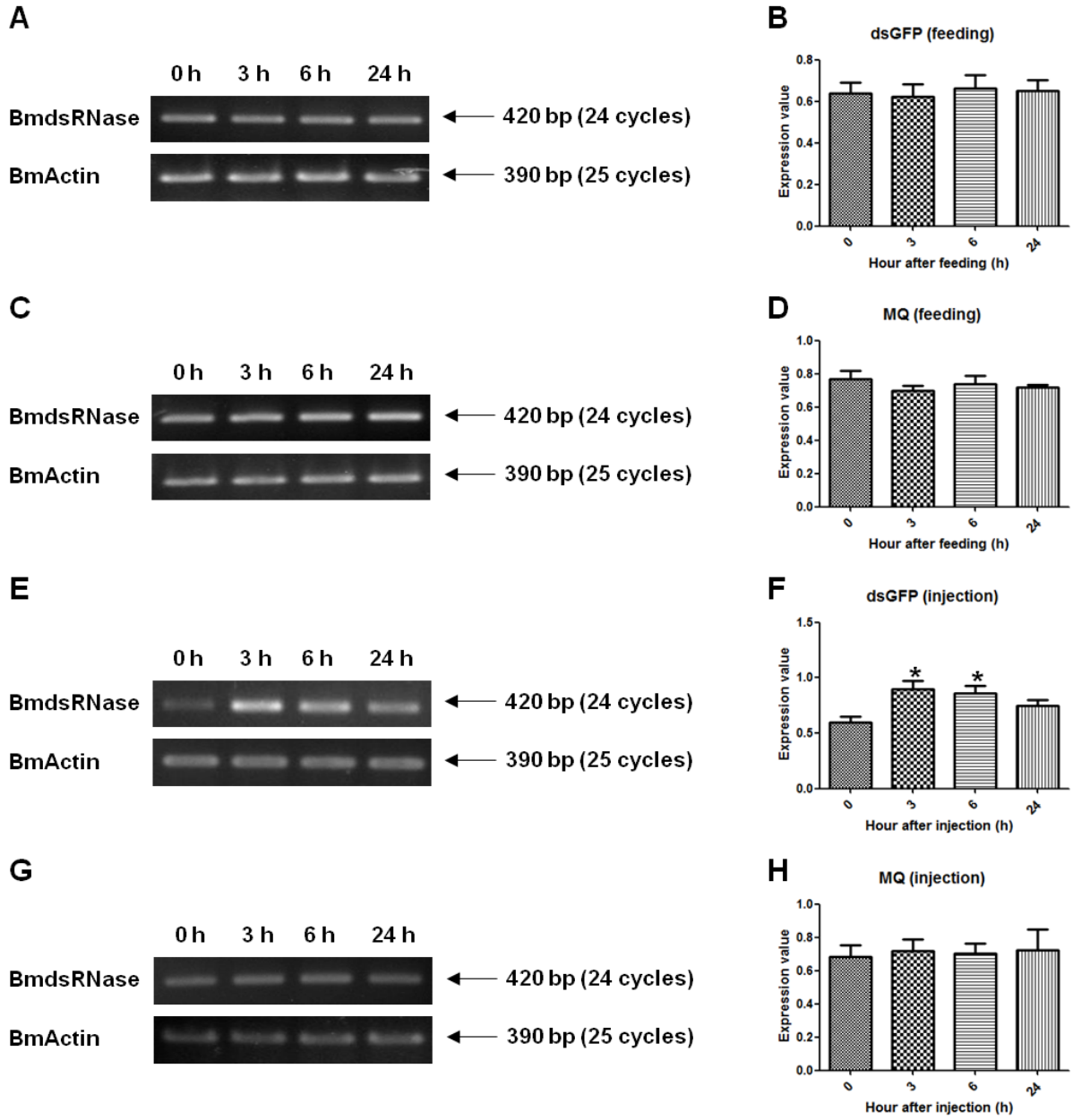


Fig. 6.6. Detection of *BmdsRNase* mRNA after feeding and injection of dsGFP. Larvae were fed with (A) 50 µg of dsGFP, (C) 100 µl of MQ or injected with (E) 5 µg of dsGFP, (G) 10 µl of MQ. Quantification of relative expression quantity of *BmdsRNase* after feeding with (B) dsGFP, (D) MQ or injection with (F) dsGFP, (H) MQ. Samples with statistically difference with respect to the control (0 h) are indicated by asterisk ($P<0.05$, $N=3$).

6.3.7 Enhanced suppression of *BmToll9-1* expression by bacteria that express dsRNA

To investigate whether dsRNA could trigger a similar transcriptional response by another delivery method, the dsGFP-producing vector pLitmus28i was introduced to the *E. coli* bacterial strain HT115(DE3). Both RNA strands corresponding to the GFP insert were

detected at high levels by RT-PCR in the transformed strain (Fig. 6.7). The non-transformed HT115(DE3) strain was used as a control. As it shows in Fig. 6.7, dsGFP was only present in the bacteria transformed with the dsGFP producing plasmid Litmus28i and IPTG was not necessary in the induction of dsGFP. Larvae were fed with the diet that was coated with these two types of bacteria.

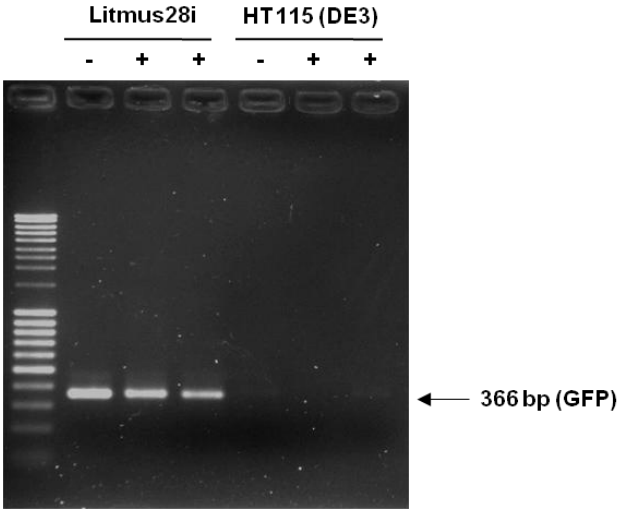


Fig. 6.7. RT-PCR detection of dsGFP frgment produced in bacteria. "-" indicated no IPTG was added, while "+" indicated IPTG wad added to induce the expression of dsGFP in the bacterial culture.

As shown in Figure 6.8C, feeding of non-transformed bacteria could down-regulate the expression of *BmToll9-1* mRNA after 6 h. Expression could still be detected in 24 h which was significantly suppressed. This demonstrated a similar expression pattern as observed in the LPS feeding assay. Meanwhile, bacteria that expressed dsGFP can suppress the expression of *BmToll9-1* after 3h (Fig. 6.8A). No expression was detected after 6 h, which indicated that dsRNA has an additive effect to suppress *BmToll9-1* expression in the midgut of *Bombyx* larvae.

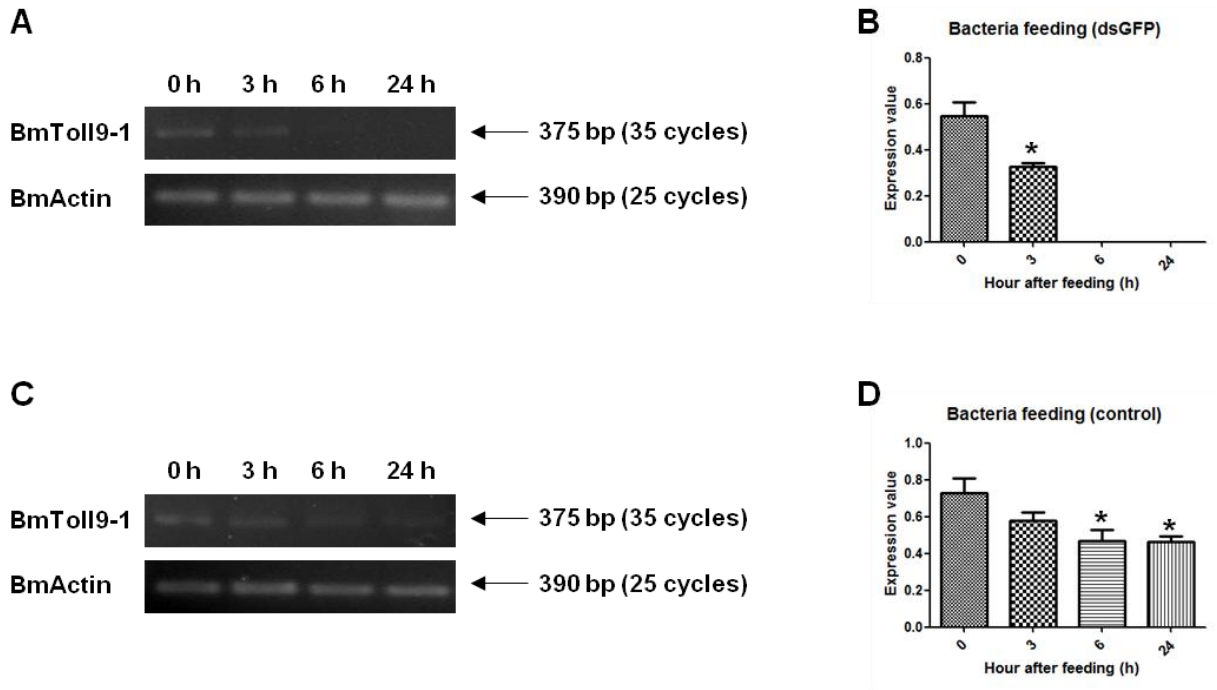


Fig. 6.8. Bacteria feeding assay to detect the expression *BmToll9-1*. (A) Bacteria with the RNAi feeding vector Litmus28i that over-expressed dsGFP and (C) bacteria without the feeding vector were coated to the diet and used in the feeding experiment for the larvae. Quantification of relative expression quantity of *BmToll9-1* after feeding with (B) bacteria over-expressing dsGFP or (D) normal bacteria strain. Samples with statistically difference with respect to the control (0 h) are indicated by asterisk ($P<0.05$, $N=3$).

6.3.8 Up-regulation of RNAi-related genes by dsRNA

In order to detect the effect of dsRNA administration on the RNAi process, the expression of the mRNAs of two RNAi core machinery genes, *BmDcr2* and *BmAgo2*, was examined by RT-PCR. MQ water was used as a negative control and it had no effect on the mRNA expression of both *BmDcr2* and *BmAgo2* (Fig. 6.9D & J). Feeding of dsGFP also did not alter the expression levels of both genes (Fig. 6.9A). However, injection of dsGFP could up-regulate the expression of both *BmDcr2* and *BmAgo2* (Fig. 6.9G). *BmDcr2* was significantly induced at 3 and 6 h after injection, where the expression folds were 2.3 at 3 h and 2.0 at 6 h compared with samples at 0 h (Fig. 6.9H). *BmAgo2* was also significantly evoked at 3 h (1.6 fold) and 6 h (1.5 fold) after injection (Fig. 6.9I). LPS did not affect the expression of both genes (data not shown).

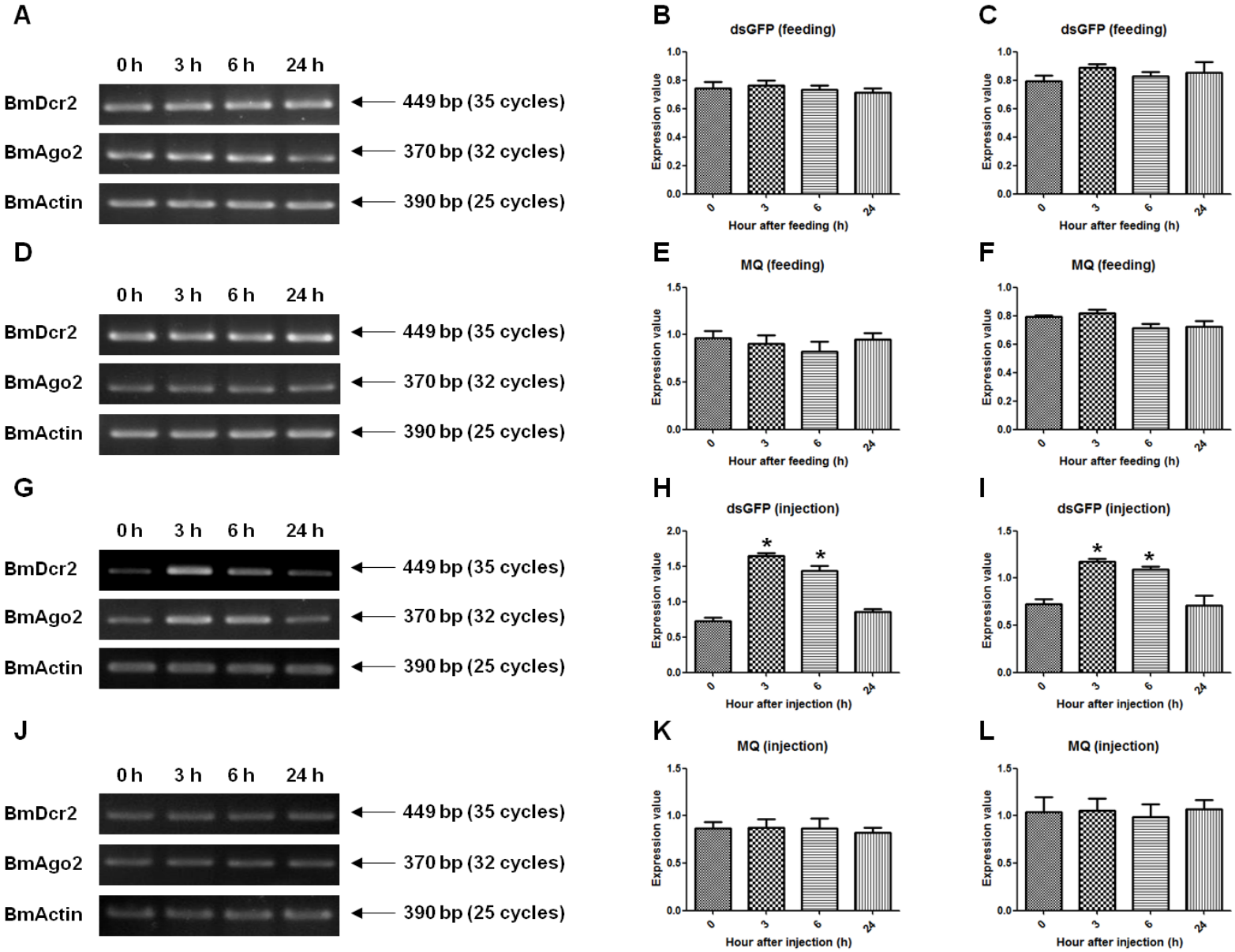

A
0 h
3 h
6 h
24 h
BmDcr2
BmAgo2
BmActin
449 bp (35 cycles)
370 bp (32 cycles)
390 bp (25 cycles)
B
dsGFP (feeding)
C
dsGFP (feeding)
D
E
MQ (feeding)
F
MQ (feeding)
G
H
dsGFP (injection)
I
dsGFP (injection)
J
K
MQ (injection)
L
MQ (injection)
Expression value
Hour after feeding (h)
Hour after injection (h)

Fig. 6.9. Detection of *BmDcr2* and *BmAgo2* mRNA after feeding and injection of dsGFP. (A) Larvae were fed with 50 µg of dsGFP, (D) 100 µl of MQ or (G) injected with 5 µg of dsGFP, (J) 10 µl of MQ. Quantification of relative expression quantity of (B) *BmDcr2* and (C) *BmAgo2* after feeding with dsGFP, (E) *BmDcr2* and (F) *BmAgo2* after feeding with MQ or (H) *BmDcr2* and (I) *BmAgo2* after injection with dsGFP, (K) *BmDcr2* and (L) *BmAgo2* after injection with MQ. Samples with statistically difference with respect to the control (0 h) are indicated by asterisk ($P<0.05$, $N=3$).

6.4 Discussion

Although there have been 14 Toll receptors identified in *Bombyx* (Tanaka et al., 2008), this is the first time to study the mRNA expression profiles of all 14 Toll receptors in larval and pupal tissues, as well as in Bm5 cells. Up to date, there are only three articles studying the expression profiles of Toll receptors in *Bombyx* (Cheng et al., 2008; Imamura and Yamakawa, 2002; Wu et al., 2010a). Through our experiments, a more complete overview of the expression profile of Toll receptors in different tissues and developmental stages is presented.

BmToll7-2 has been reported to be expressed only in the fat body (Imamura and Yamakawa, 2002), but our results demonstrated that its expression was not restricted to the fat body only but very broad expression in all other larval tissues was observed (Fig. 6.1). In another study (Cheng et al., 2008), microarray was used to analyze the expression profiles of 13 Toll receptors except *BmToll9-1*. In their research, the expression pattern of the 13 Toll receptors was similar with our results, but there still exist some differences. They also could not detect the expression of *BmToll7-2* in the midgut, while we found that *BmToll7-2* was widely expressed. Their results also indicated that *BmToll3-2* was not expressed in the gonads, while the mRNA of *BmToll3-2* was relatively abundant in our experiments. A common element in our and their study was that the expression levels in the immune organs, fat body and hemocytes, were low to absent. The above-mentioned differences are likely to be explained by different times of collection of tissue, the use of different silkmoth strains and the use of different methodology. The third experiment was performed in 5^{th} instar larvae at day 3 (Wu et al., 2010a), in which expression of *BmToll9-1* was studied. They found that the expression of *Toll9-1* was strong in different parts of the gut, which was consistent with our results that *BmToll9-1* had higher expression in the midgut than in other tissues (Fig. 6.1).

When a phylogenetic tree was generated to compare the evolutionary relationship of the 14 *Bombyx* Toll receptors with the mammalian Toll-like receptors, it was found that BmToll9-1 and BmToll9-2 are more closely related to HsTLR3 (dsRNA as ligand) and HsTLR4 (LPS as ligand) (Alexopoulou et al., 2001; Tsukamoto et al., 2012), respectively,

than to the other receptors (Fig. 6.2B & C). However, the homology between BmToll9-1 and HsTLR3 or between BmToll9-2 and HsTLR4 is quite low with less than 20% of similarity. Thus, from the phylogenetic analysis it is not clear at all whether BmToll9-1/BmToll9-2 can interact directly with dsRNA/LPS.

Furthermore, in the phylogenetic tree BmToll9-1 forms a subgroup with other Toll9 receptors from other insect species (Fig. 6.2A). A study indicated that *Drosophila* Toll9 could trigger a similar immune response as Toll (the established receptor involved in innate immunity against Gram-positive bacteria and fungi with Spätzle as ligand) by establishing a constitutive antimicrobial defense in S2 cells (Ooi et al., 2002). In *Anopheles gambiae*, AgToll9, which had strong expression in the gut, was also suggested to play a role in the immune response (Christophides et al., 2002). Since BmToll9-1 is related to DmToll9 and AgToll9 and its dominant expression is also in the midgut, it is more likely to be involved in the immune response than to have a pure developmental role.

Larval gut contains LPS and other pathogens by feeding from the diet, which might explain the strong expression of BmToll9-1 in the gut. However, most of the studies regarding the immune pathways mainly focus on the transcription of AMPs. It is hard to explain the mechanism of this down-regulation by LPS. LPS injection to the hemocoel was performed in *Bombyx* larvae to detect the expression of *BmToll7-2* after injection (Imamura and Yamakawa, 2002). They found that *BmToll7-2* was strongly down-regulated by LPS injection. This result is consistent with our experiments in which LPS also suppressed the expression of *BmToll9-1* via feeding and injection of LPS (Fig. 6.3 & 6.4). While our experiments showed a transcriptional response of *BmToll9-1* to LPS in the midgut, these data cannot prove that LPS (or dsRNA; discussed later) directly interacts with BmToll9-1 protein. However, LPS can function as a PAMP to trigger the innate immune response in insects which is reflected in the induction of immunity genes. Our data therefore indicate that the decrease in *BmToll9-1* mRNA is part of the immune reaction triggered by LPS.

Besides LPS, dsRNA injection could also trigger the down-regulation of *BmToll9-1*, starting after 3 h (Fig. 6.3B). However, dsRNA feeding did not significantly suppress the expression of *BmToll9-1* (Fig. 6.3A). Also the expression of *dsRNase*, *Dcr2* and *Ago2* did not change in the dsRNA-feeding experiment, while in the injection experiment the expression of these genes was triggered. One of the differences between the two dsRNA-delivery methods may be the presence of abundant dsRNA-degrading enzymes such as BmdsRNase (Arimatsu et al., 2007b) in the midgut content. In our previous study, the mature type of BmdsRNase could efficiently degrade dsRNA in the Bm5 cells (Liu et al., 2012). In order to protect

dsRNA from degradation by dsRNA-degrading enzymes, 24 h starvation was executed prior to feeding assay, which results in significant reduction in nuclease activity in *Manduca sexta* (Rodriguez-Cabrera et al., 2010). However, dsRNA was still quickly degraded by fresh midgut juice in less than 30 min. Meanwhile, dsRNA was more stable in the hemolymph than in the midgut juice (Fig. 6.5). The difference in abundance of dsRNA-degrading enzymes could therefore at least partly explain why dsRNA can trigger transcriptional responses after injection but not after feeding. Another difference between feeding and injection is also the timing of delivery. During feeding, dsRNA is taken up gradually, presumably over a period of minutes to hours, while during injection dsRNA is delivered instantly in one dose. Because of its gradual passage through the midgut, dsRNA is exposed much longer to enzymes before reaching its target.

Feeding of *E. coli* bacteria could suppress the expression of *BmToll9-1* mRNA (Fig. 6.8), a similar pattern as in the LPS feeding assay. Because LPS is a major conserved cell wall component of Gram-negative bacteria such as *E. coli*, it is not surprising that feeding of *E. coli* bacteria could also inhibit the expression of *BmToll9-1*. For bacteria that constantly express dsRNA (dsGFP), this inhibition was clearer and more instant, which confirms that dsRNA can inhibit the expression of *BmToll9-1*. Thus, bacteria can be used as carriers to keep dsRNA intact to reach its target during feeding, a strategy that has been implemented in other studies. It is therefore anticipated that bacteria producing dsRNA could also induce expression of RNAi machinery genes, a response that is observed for dsRNA injection but not for LPS administration (Fig. 6.9).

In mammals, dsRNA can be recognized as a viral molecular pattern (DeWitte-Orr and Mossman, 2010) and is able to evoke the innate immune reaction (Berke et al., 2012). Human TLR3 functions as a PRR for dsRNA that activates the NF-κB pathway and the production of type-I interferons (IFN-α/β) (Alexopoulou et al., 2001; Doyle et al., 2003). In insects, on the other hand, very little is known about dsRNA as a potential trigger of the innate immune response. In *Drosophila*, the Toll pathway is important for the antiviral response and the suppression of the replication of *Drosophila* X virus (DXV), whose genome has a dsRNA structure (Zambon et al., 2005). In this study, dsRNA can induce a similar transcriptional response as LPS with respect to regulation of *BmToll9-1*. This observation suggests that it can also act as a PAMP in *B. mori*. Furthermore, dsRNA can induce the expression of RNAi machinery core genes and *dsRNase*, which can be regarded as a defense mechanism. These data suggest that dsRNA can trigger an immune reaction in insects. However, there is no evidence that the BmToll9-1 receptor can function as a PRR for dsRNA (or LPS). Instead,

BmToll9-1 likely functions in the transcriptional cascade induced by the PAMPs, LPS and dsRNA, while their PRRs remain unidentified.

On the other hand, dsRNA is extensively used in RNAi experiments to trigger gene silencing. In Lepidoptera, RNAi efficiency shows considerable variation among different species, tissues, stages and target genes (Terenius et al., 2011). Our study indicates that dsRNA can not only influence the expression of core RNAi machinery genes but also immunity-related genes such as *BmToll9-1*. This may be a factor to explain the variability in success of RNAi experiments in Lepidoptera.

In our experiments, dsGFP was used as the non-specific dsRNA to test the response of BmToll9-1 and RNAi machinery genes. dsGFP is generally used as negative control dsRNA in RNAi experiments in insects (Terenius et al., 2011). The sequence of the dsGFP fragment (494 bp) does not show any obvious motifs or secondary structures that would cause a different behavior than other dsRNAs. However, future studies could be carried out to determine whether certain lengths or sequences of dsRNA are more or less potent to trigger the transcriptional response reported in this study. It is noted that in another study that determined the transcriptional response of RNAi machinery genes in *M. sexta*, dsGFP was also used (Garbutt and Reynolds, 2012).

In summary, while the role of Toll receptors functioning in immunity in *Bombyx*, is not all well clarified so far, our study represents the first example to study the response of *BmToll9-1* to dsRNA in silkmoth larvae. The exposure to dsRNA can trigger the down-regulation of *BmToll9-1*, a similar effect as observed for LPS. Since *BmToll9-1* was dominantly expressed in the midgut which is the first layer of defense, BmToll9-1 might be involved in the immune response as other Toll9 receptors in *Drosophila* and mosquito. The specific induction of *BmDcr2* and *BmAgo2* could also contribute to the immune responses in the insects against pathogens carrying dsRNA structures (*e.g.* RNA viruses).

Chapter 7

Modulation of innate immune genes and RNAi machinery genes in silkmoth-derived Bm5 cells following expression of BmToll9-1 receptor[5]

[5] Adapted from

Jisheng Liu, Anna Kolliopoulou, Guy Smagghe, Luc Swevers (2013). Modulation of the transcriptional response of innate immune and RNAi genes to dsRNA and LPS in silkmoth-derived Bm5 cells following expression of BmToll9-1 receptor.

7.1 Introduction

Insects first appeared on earth approximately 350-400 million years ago and they are composed of more than 70% of the animal species today (Mayhew, 2007). One of the reasons for this successful evolution is the effective innate immune system in the insects. Unlike the vertebrate animals that have innate and acquired immune systems, insects only have the innate immune system which has been shown to respond to bacteria, fungi, parasites and viruses. (Evans et al., 2006).

There are several signal transduction pathways in insects induced by pathogens (Merkling and van Rij, 2012), which include Toll pathway, immune deficiency (IMD) pathway, Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway and c-Jun N-terminal kinase (JNK) pathway (Merkling and van Rij, 2012; Tanaka et al., 2008). These inducible immune pathways allow insects to rapidly respond to the invading pathogens, such as bacteria, fungi and viruses, after recognition by receptors on the cellular membranes. This recognition immediately activates respective immune signal pathways and triggers the transcription of downstream genes afterwards, which leads to the release of the effector molecules, especially the antimicrobial peptides (AMPs) which become highly expressed in the fat body and hemocytes (Christophides et al., 2002; Ferrandon et al., 2007; Lemaitre, 2004; Lemaitre and Hoffmann, 2007; Reichhart et al., 1993; Tauszig et al., 2000).

Besides the above-mentioned signaling transduction pathways, RNA interference (RNAi) is also regarded in the context of the antiviral immune response, which causes gene specific silencing (Karpala et al., 2005). In this process, dsRNAs or viral genomes with dsRNA structure are cleaved by Dicer enzymes into small interfering RNAs (siRNAs) that are in turn loaded into RNA-induced silencing complexes (RISCs) that scan RNAs for complementary sequences and degrade mRNA and viral RNA (Carthew and Sontheimer, 2009; Siomi and Siomi, 2009).

The signal transduction pathways and induction mechanism of AMPs have been extensively studied in *Drosophila melanogaster* (Aggarwal and Silverman, 2008; Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). In *Drosophila*, activation of transcription of AMP genes is under the control of two NFκ-B signalling cascades, IMD pathway which is activated by Gram-negative bacteria, and the Toll pathway which is activated by fungi and Gram-positive bacteria (Hetru and Hoffmann, 2009). Imd and Toll pathways resemble closely the tumor necrosis factor-receptor (TNF-R) and Toll-like receptor (TLR) pathways, respectively in mammals. However, while in mammals TLRs recognize directly pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), dsRNA, Flagellin

and unmethylated CpG DNA (Kawai and Akira, 2010), the *Drosophila* Toll receptor essentially functions as a cytokine receptor with the polypeptide Spätzle as ligand (Michel et al., 2001; Weber et al., 2003). Activation of the Toll receptor by bacterial peptidoglycan (PGN) occurs through binding to the secreted protein peptidoglycan recognition receptor SA (PGRP-SA) which triggers an extracellular proteolytic cascade that ultimately transforms pro-Spätzle into an active ligand for Toll (Kawai and Akira, 2010; Michel et al., 2001; Weber et al., 2003).

While Toll (or Toll-1) has a clear role in the innate immune response, the functions of the 8 other Toll receptor genes that were identified in the *Drosophila* genome are not as well established (Ferrandon et al., 2004; Narbonne-Reveau et al., 2011). Nevertheless, indications exist in literature that other Toll receptors have a function in the immune response in *Drosophila* (Bettencourt et al., 2004; Nakamoto et al., 2012; Ooi et al., 2002), although the prevailing view is that most other Toll receptors have a developmental role (Ferrandon et al., 2004; Kambris et al., 2002). Interestingly, phylogenetic analysis revealed two clades of Toll receptors; the first one being composed of most insect Toll receptors while the second one groups the mammalian TLRs with Toll-9 (Bilak et al., 2003; Ferrandon et al., 2004). Toll-9 has also the same ectodomain organization as mammalian TLRs and it has therefore been speculated that Toll-9 could function as a PRR, similar to mammalian TLRs.

In the silkmoth, *Bombyx mori*, 14 Toll-related genes were identified (Liu et al., 2013; Tanaka et al., 2008). Of relevance is that Toll-9 is conserved among insects (Liu et al., 2013; Luna et al., 2002; Wu et al., 2010a). While BLAST searches identify two Toll-related receptors in *Bombyx* as closely related to *Drosophila* Toll-9, further analysis has revealed that BmToll9-1 constitutes the true ortholog and that BmToll9-2 is more distantly related (Liu et al., 2013; Wu et al., 2010a). In our previous study (Liu et al., 2013), we found, first, that *BmToll9-1* was dominantly expressed in the larval midgut as the first barrier of defense against invading pathogens, and second, that administration of LPS and dsRNA caused the transcriptional response of *BmToll9-1*, indicating its possible role in immune response. The dominant expression of Toll-9 in gut tissues was also observed in mosquitoes (Luna et al., 2002). Given the structural properties of the Toll-9 receptor, its preferential expression in gut tissue and its transcriptional response to administration of LPS and dsRNA, it was decided to study the functional properties of BmToll9-1 in more detail. Because *BmToll9-1* was hardly expressed in the Bm5 cells (Liu et al., 2013) and Bm5 cells can be efficiently engineered to express foreign proteins (Douris et al., 2006), they could function as an experimental system to investigate the involvement of BmToll9-1 receptor in the innate immune pathways and

RNAi pathway.

In this chapter, sequences of the cDNA of BmToll9-1 corresponding to the full-length protein and the ectodomain (ECD) were cloned in expression vectors and analyzed for expression in Bm5 cells by Western blot and immunostaining for localization. The interaction between BmToll9-1 receptor and dsRNA was investigated by studying the binding activity of BmToll9-1 and the modulation of the RNAi response following over-expression of BmToll9-1 protein in Bm5 cells. Finally, BmToll9-1 expressing and control cells were soaked with dsRNA and LPS, and we followed the transcriptional response of selected genes in the immune signaling and RNAi pathways.

7.2 Material and methods

7.2.1 Experimental animals

The larvae of *B. mori*, Daizo strain, were reared on artificial diet (Yakuruto Co., Japan) at 25°C under a photoperiod of 12 h light and 12 h darkness. Midgut tissues from 3-10 larvae were dissected from 5^{th} instar larvae at day 4-5 after the molt, collected in eppendorf tubes on ice and frozen at -70ºC until further processing.

7.2.2. Full length cloning and bioinformatic analysis of BmToll9-1

Frozen tissues were homogenized in TRI Reagent (Sigma) and total RNA was extracted according to the manufacturer's protocol. The quantity of extracted RNA was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and/or by electrophoresis on 1% (w/v) agarose gels. First-strand cDNA from 1 µg RNA was synthesized using a SuperScript II reverse transcriptase (Invitrogen) and a $Oligo(dT)_{12-18}$ primer (Invitrogen) following program: 25°C for 10 min, 42°C for 1 h.

In order to clone the full length of BmToll9-1, the amino acid sequence of BmToll9-1 (Tanaka et al., 2008) was used to search against the *Bombyx* genomic database, KAIKOBLAST. An upstream in-frame ATG is taken as the translation start, while a downstream in-frame TAA is taken as the translation stop codon. Primers (forward: 5'-AAATCGCCTCTCCGTTTAGTGTCC -3'; reverse: 5'-CGAGTCGTATTCACCTTGTGGTCCTA -3') used to amplify the open reading frame (ORF) were designed in the untranslated regions (UTRs). The PCR program was performed as follows: 94°C denaturation for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 140 s for each cycle, and a final elongation step at 72°C for 10 min.

The full length of BmToll9-1 was analyzed by SignalP4.0 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) to predict the signal peptide and transmembrane domains.

7.2.3. Expression and reporter constructs

The complete ORF of BmToll9-1 was amplified with the forward primer 5'-GCGC*GGATCC*CACCATGATATTGAAGATCATTAAGC -3' and the reverse primer 5'-ATGC*GGATCC*AGCTAAAGATACGTTTTCCG -3', the PCR fragment was digested with BamHI (in italics in the primer sequence) and cloned into the BamHI-site of the pEA-MycHis vector (Douris et al., 2006). The reverse primer is designed to allow the in-frame fusion with a MycHis-tag in the expression vector, to generate plasmid pEA-BmToll9-1-full-MycHis. The same forward primer was also used in combination with the reverse primer 5'-GCGC*GGATCC*TTTGTACTGATTTTTAATCATC -3' to clone the ECD of BmToll9-1 and generate the expression plasmid pEA-BmToll9-1-ECD-MycHis.

In order to express the LRRs-containing ECD of BmToll9-1 in *Escherichia coli* bacteria, the forward primer 5'-ATAC*GGATCC*CGAGGAAAACACAAAAATGC-3' and the reverse primer 5'-GCGC*GGATCC*TCATTTGTACTGATTTTTAATCATC-3' were used to amplify this region. Then the PCR fragment was digested with BamHI (in italics in the primer sequence) and cloned into the BamHI-site of the pGEX-5X-3 expression vector (GE Healthcare), in-frame with the glutathione-*S*-transferase (GST) ORF.

7.2.4. Cell lines, transfections and soaking

Bm5 cells were maintained in IPL-41 medium (Gibco) supplemented with 10% FBS and grown at 27°C. Transfection was according to established protocols (Johnson et al., 1992). For expression studies, 1 μg/ml of pEA-BmToll9-1 expression vector (described above) was used together with 0.5 μg/ml of pBmIE1 helper plasmid encoding the ie-1 gene from BmNPV (Lu et al., 1997). Cells were harvested at 2-3 days after transfection for expression analysis or functional assays.

To generate a transformed Bm5 cell line that permanently over-expresses BmToll9-1-full, cells were transfected as above but in the presence of 150 ng/ml of pEA-PAC vector, which confers resistance to the antibiotic puromycin. Resistant cells that over-express BmToll9-1 were selected at 20 μg/ml puromycin as described (Douris et al., 2006). Another

transformed Bm5 cell line that contains pEA, the empty expression vector, was generated as a control cell line (Bm5-pEA cell line).

Transformed Bm5 cells (Bm5-pEA-BmToll9-1 and Bm5-pEA) were soaked with 5 μg/ml dsGFP or LPS, respectively. After 48 h of soaking, cells were collected by low speed centrifugation (800 g, 5 min).

7.2.5. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted with TRI Reagent and cDNA was synthesized using SuperScript II reverse transcriptase. qPCR was performed with SsoFast ™ EvaGreen ® Supermix (Bio-Rad) to detect the expression of target genes (primers are described in Table 7.1). To carry out qPCR, 500 nM of specific primers and 20 ng of cDNA were used in a total volume of 20 μl. Translation initiation factor 4A (TIF4A) was used as the reference gene (Wang et al., 2008). The thermal profile for amplification was as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 s and 60°C for 60 s in CFX96 ™ Real - Time PCR Detection Systems (Bio-Rad). Relative expression levels of the target genes (X) were determined as ratios to the transcripts of the reference gene (R), as the $2^{-\Delta\Delta Ct}$ method, with $\Delta Ct = (Ct(X) - Ct(R))$ (Livak and Schmittgen, 2001). All qPCR reactions were performed as triplicates for the soaking experiments with LPS and dsRNA for both Toll9-1-expressing and control transformed cell lines. The cell lines after soaking were compared with the cell lines without soaking to calculate the induction by dsRNA and LPS. All the calculated values were statistically analyzed by one-way ANOVA. Statistic differences between BmToll9-1 and control cell line was also analyzed with one-way ANOVA.

7.2.6. Protein extracts preparation and Western blot analysis

Transfected cells cultures were collected and separated as extracellular medium, soluble cellular extracts and insoluble cellular pellets by centrifugation and freeze-thawing as described (Liu et al., 2012). Ten μg of proteins were run with 10% Bis-Tris NuPAGE Gel (Invitrogen) in MOPS Running Buffer (Invitrogen). Then the proteins were immediately transferred to PVDF membrane (Invitrogen) for Western blot analysis. Primary antibody used was mouse anti-Myc at 1:1000 (Cell Signalling). The secondary HRP-conjugated anti-mouse antibody (Cell Signalling) was used at 1:1000. Then, 3,3'- diaminobenzidine tetrahydrochloride hydrate (Sigma) was used as substrate for development (Liu et al., 2012).

Table 7.1. Primer sets used for qPCR. Abbreviations of AMPs: Att = Attacin, CecE = Cecropin E, Def = Defensin, Leb = Lebocin, Mor = Moricin, Lys = Lysozyme, Hem = Hemolin.

Genes	Accession number	Primer pair	Size (bp)
Toll pathway:			
MyD88	XM_004921515	TAATAGACTCGGAGGAATG CGATACTAATAGCCTGTGC	191
Rel	AB096087	CCAGCAAAGCCCTCAGA GTTGTGCGGGTGCGGTT	185
JAK/STAT pathway:			
Dome		TTACACTGGCATTGAACACC TGAATCACTAAGCACATCGG	
Stat	NM_001163916	GCCAGGTGTTGGACGATGA CACGATGGTCTTGCTGTTGAT	182
IMD pathway:			
Imd	XM_004930229	AGCCGGAAGATGAGCATTTA GTATCAATTCTGCCTTCGGTGA	100
Relish	AB298441	TTCGGTGGAATGGGTATCAT GCTGAACTTCAAACGCACAA	165
Tak1	GQ426306	TAATGGCCGCTGAGGCGCAC ACGGGCGAGCTCGATGTCCT	169
AMPs:			
Att	NM_001043541	TGCTTGGCGAGCGGTAGA GCCCGTGCCCGTTTACAT	220
CecE	D17394	CTTCGTCTTCGCGTTGGT AAGGATTTCGCTTGCCCTAT	159
Def	NM_001043905	GGTGCTCGTGTTTGTGTTTG CAAATCGCAGTCTCTGTTGC	165
Leb	NM_001126260	ACACGTACAGTGCGACAAGC CAATGGACGCCTCGTTATTT	184
Mor	AB006915	TCTTTGTTTTTATTGTGGCAATG TTGAAAACATCGTTGGCTGT	151
Lys	L37416	TTGGTTGTCCTCTGCGTTGG CGGTTCGTGTTCGTCTTGG	161
Hem	FJ911550	GGGAGGAGACTGGTCATCAA GGAATTCCTGTCGCCTTACA	88
RNAi pathway:			
Dcr2	NM_001193614	AAATGACTGACCTGAGATCCGC AGCAGAGGTACTTGTGCAATCC	87
Ago2	NM_001043530	CGAAGCTAATGAATACCGGTCG CGTGCAGCATACTCTTGTGCA	105
Ago3	NM_001104597	TCGAGAGACGGCTACGACAAT CTGCGAGACGATCAGGAAATC	93
Reference gene:			
BmTIF4A	DQ443290	TTCGTACTGGCTCTTCTCGT CAAAGTTGATAGCAATTCCCT	174

7.2.7. Immuno-fluorescence microscopy

Immuno-fluorescent staining of Bm5 cells with specific antibodies was carried out as described before (Labropoulou et al., 2008). In brief, cells were initially stained with 5 μg/ml of Texas Red®-X conjugated wheat germ agglutinin (WGA, Invitrogen) or 1 mM of LysoTracker® (Invitrogen) for 10 min in PBS at 28°C for the co-staining experiments. Then the cells were fixed with 4% formaldehyde in PBS for 20 min and subsequently permeabilized with PBS supplemented with 0.1% (v/v) Triton X-100 (PBS-T) for 10 min. Cells were stained overnight at 4°C with mouse anti-myc antibody (Cell Signalling) or rabbit anti-Flag antibody (Sigma) at 1:200. Following five washes in PBS, FITC-conjugated goat anti-mouse secondary antibody (Sigma; at 1:200) or AlexaFluor-labeled anti-rabbit antibody (Molecular Probes; at 1:500) were added for 1 h at room temperature. Cells were again extensively washed with PBS, stained with DAPI (1 μg/ml, 5 min), mounted in Mowiol 4-88 (Sigma) and examined under a fluorescence microscope (Zeiss Axiovert 25 inverted microscope). For confocal microscopy, the cells were treated as described above and observed in a BioRad confocal microscope (MRC 1024 ES) equipped with Lasersharp software (Bio-Rad) and a kryptonargon laser. Confocal microscope data were processed using the GNU Image Manipulation Program (GNU Project, http://www.gnu.org/).

7.2.8. Expression and purification of GST fusion proteins in bacteria

The pGEX-5X-3 expression vector containing the LRR fragment was introduced into *E. coli* DH5α bacteria. The pGEX-5X-3 vector containing the ORF of Flag-tagged B2 protein, a dsRNA-binding protein from a nodavirus that persistently infects Hi5 cells (Li et al., 2007) was used as a binding positive control, and the empty vector that expresses GST was used as a binding negative control. Expression of the target proteins was performed according to the "GST Gene Fusion System Handbook" (GE Healthcare).

Bacteria pellets were collect by centrifugation at 10,000 g for 5 min and resuspended with ice-cold PBS supplemented with 1.5% sarcosyl, 1 mM PMSF, 5 mM DTT, which was incubated on ice for 30 min. Then the bacteria suspension was sonicated at settings of 60% duty cycle, power 6, 30 s each pause, for a total of 3 min (UP200s sonicator; Hielscher). Triton X-100 was added to a final concentration of 2% to the post-sonicate which was kept on ice for 30 min. The lysate was centrifuged at 12,000 g for 10 min at 4°C. Then the supernatants were collected and 50% slurry of Glutathione Sepharose 4B (GE Healthcare) was added to the supernatants, and gently mixed at room temperature for 60 min. Glutathione Sepharose 4B beads were sedimented by centrifugation at 500 g for 5 min and the beads were

washed with ice-cold PBS for three times. Then the beads were analyzed for the presence of dsRNA by RT-PCR.

7.2.9. dsRNA binding activity assay

dsRNA corresponding to GFP (dsGFP) was synthesized according to (Liu et al., 2012). dsRNA binding assay was carried out according to (Fenner et al., 2006) with small modifications. One microgram of dsGFP was incubated in 500 µl of 50% slurry binding with GST fusion protein (20 µg of GST, 3 µg of GST- Flag-B2, 1 µg of GST-BmToll9-1-ECD) in binding buffer (100 mM of NaCl, 50 mM of Tris-HCl; pH 7.4). The reaction was incubated at room temperature for 1 h. Afterwards, the beads were collected by centrifugation and washed with 1 ml of PBS for 3 times. Three micrograms of tRNA (Sigma) were added as carrier prior to the extraction of RNA using Trizol. First strand cDNA from 500 ng extracted RNA was synthesized with SuperScript II reverse transcriptase and dsGFP-specific primer (5'-CTACCCCGACCACATGAAGC-3'). PCR was performed with the forward primer (5'-GCTTCTCGTTGGGGTCTTTG-3') and reverse primer (5'-TCCAGGAGCGCACCATCTTC-3') to detect dsGFP. The PCR amplification program was at 94°C 30 s, 55°C 30 s, 72°C 25 s, for 30 cycles.

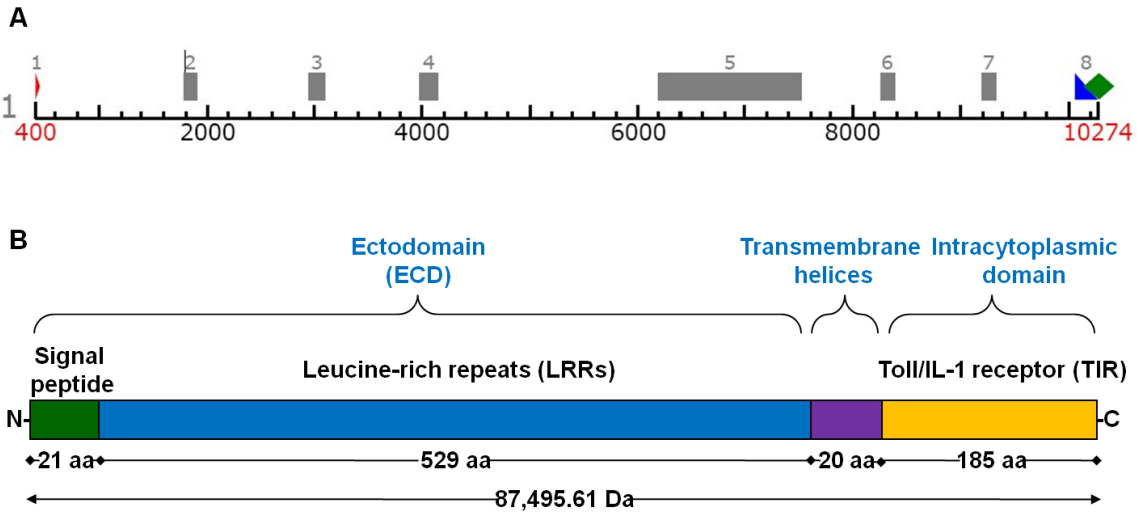
7.2.10. RNAi response assay in Bm5 cells

RNAi experiments were carried out as described before (Swevers et al., 2011). Bm5 cells were transfected with 0.9 µg/ml of pERE-Luc luciferase reporter, 0.2 µg/ml of pERE-gfp fluorescence reporter, 0.9 µg/ml of expression plasmid pEA-PAC (negative control; expresses puromycin N-acetyl transferase) or pEA- BmToll9-1-full-MycHis, and 0.05 µg/ml of dsRNA (specific dsLuc dsRNA derived from the luciferase ORF (Swevers et al., 2011), or non-specific dsMalE as a negative control (Zhu et al., 2011)). Two days after transfection, the ecdysone agonist tebufenozide was added at 500 nM to induce the ecdysone reporter genes pERE-luc and pERE-gfp. Soluble cellular extracts of transfected cell populations were prepared as described (Swevers et al., 2011) and directly used for fluorescence measurements or processed for luminescence measurements using the Steady-Glo® Luciferase Assay System kit (Promega) according to the manufacturer's instructions. Specific luciferase activities were calculated as luminescence/fluorescence ratios. Both fluorescence and luminescence measurements were carried out with an Infinite M200 luminometer (Tecan, Switzerland) (Soin et al., 2010). Statistical analysis of differences in normalized luciferase activity was carried out by one-way ANOVA analysis.

7.3 Results

7.3.1 Identification of BmToll9-1 in the *Bombyx* genome and expression Bm5 cells

During our search against the KAIKOBLAST database, BmToll9-1 was located in Scaffold 12 in the *B. mori* genome sequence. The gene of *BmToll9-1*, encompassing start and stop codons in the ORF, is >10 kb in length. Gene structure analysis showed that *BmToll9-1* consists of 8 exons and 7 introns (Fig. 7.1A). The length of BmToll9-1 cDNA, from start to stop codon, is 2268 bp (Supplementary text). Protein structure analysis showed that BmToll9-1 is composed of a signal peptide (21 aa), an LRRs-containing ECD (529 aa), one transmembrane helix (20 aa) and a cytoplasmic Toll/IL-1 receptor signaling domain (TIR, 185 aa) (Fig. 7.1B).



C

ATGATATTGAAGATCATTAAGCTATTAAAATTCTTTCTGCTCTTCATCGCATCAGCGGAAACGAGGAAAACACAAA
AATGCTTAACCGGCTACATGACGGACGTTCAAACCTGGGTGAACGAAGATGGCGTTCTCACGAAAACAATCGATC
AAAGCACCGCTATAGATCTTTCTTCGCACTTCGATCCGGTCCCATTCCTTCATCAGATCATGAGTGAATTCAAAACG
GACAATGGGCAACGCGTCCGATATTTAAGCCTGGCGAAATGTCGACTCACCAGAGTGCCACCTGTATTCCATTTGT
CAGACTCAATGGGACGTTGGTTATCGCAGACCGTTGAGTACATGACTTTCTACGGAAATAAATTCGTCGACAGAA
GCGTGGCCGGTGAACAGTACAACATGATTATAAACGCTACCGGCGCTGTCGAAACTGAGCCGGTGTTCAGCTTAC
AGTACCAAGGTACGACAGTCTGGTCCACTTCAATTGAATACGTCAGCTTCCCGAGCCTAAAGGAGCTGGATCTGCG
AAGGTGTTCAATCCAAGTGCTCAGAAGCAATATGTTCAGGGGTATGCCTTCACTAGAAGCTTTATATATAGGGGA
GAATGAGATTTATCAAGTCGAATCCAACACTTTTGCTGGTCTAAATAAGTTATTACATCTAGACTTTAGCAGGAACG
AAGCATTCGACAGTACGGGGTCTCCTAAAAATCTAATTACCCCGCAAACACCGCTATTCGATGGCCTAACAAGTTT
GGTATCTCTAGATCTTTCGTTCACAAAGATGTCCAAACGTAATGTGGCAGTGCTAACAGGCTTAGGACCGAATTTG
AGAAGGCTGTCGATATGCGATACAGGGCTGCAGGATTTGAGGAGCGACATTTTTTCTAAAACAAATCTCACGTACT
TGGATCTTTCGGAGAACAACGGGATCTTGAACACGCCTAACATATTGCGAGGGCTTGAAGATACTTTAGTGGTATT
GTACGCTAATACTATAGGCACAGGTCGGGTCGATATGTTAAAGAATTTCCACAGACTGGAGATATTGCAATTACTG
AACAACGAAATAACGTCTCTACCACAAGAAGTCGCGGCAACTTGGAGGAGCCTCCAGATTTTGGACTTGAACAAG
AACAGGATTATAACGTGGTTCGAACCGATATTCTCGCTGATACCTAACCTCAAATTCTTGGCACTCAAACACAACAA
CATAAACGTGATCCGCGAAGACATGATCGTGGATTTCAGGAACATATCGTATCTGGCCCTGGCCGGAAACTTCTTC
ATGTGCAACTGCAACTCCAGAGATTTCCTCGAGACGGCGGCTAGGAACGAGAGAAACCGACAAAACGGATATATA
AAGTCTGTTTACGAATCGAAAAACCTGTTTCTGTACCATAGAGGGTTCGAAGATTTCAATACGCTTATAGCGCAAA
GGAAGCCCGTAGTTTTCGAAAACTCCATCAGCGAATGGGACAACGAGGAAGAGAGTGTATACCTATTGACCGATT
TCTATGGTCGAAACTATATTTGTGCGTCGTTTGCTTCGGACGAAGGCGAGGCCATCTTCATGGGGGACGTGCCCAC
CTGCTACGCTCGTCGAGATATGCAGTACGACGAAAAGATGATTAAAAATCAGTACAAATTGATTGCTTTAATTCTG
TTACCGTGCGTTTTATTACCGCTGCTGATGCTGTTCGTATTCAGAAGGACGATTTTTTATTTTCTAATAATGATGCGG
AATTCTGCTGCCCTCACCATGATCAATAAGGATAAGACCGGTGTCGATGGTACAATATTCAACTACGATGTATTCG
TGTCGTACTGCAACGAGGACAGGGTGTGGGTGCTCGACCATCTCCTGCCGCAGTTGGAGTCCAATTGCAACATCA
GCGTCTGCCTCCATGAGAGAGATTTTCAGATCGGACTCTCCATCCTGGAGAATATCGTCGCCTGCATGGACCGTTC
CAGGGCGATCATGCTGATCATATCAAAACGTTTCCTCATGAGTCAGTGGTGCCAGTTCGAAATGCATTTAGCCCAG
CACAGGCTGTTAGAGACGCGTCGGAACGATTTGATACTTGTTCTGTTAGAAGAAATACCGCGGCGCATCCGCCCA
ACGACCCTCCACTATCTGATGCTGACGAAGACTTACATAATCTGGCCGAAAGTCGCGCACGAAAGAAACATTTTCT
GGAGGAGGCTACGAAAAGGCCTGGTGACGCAGAAACTGAAGCACACGGAAAACGTATCTTTAGCTTGA

Fig. 7.1. Identification of BmToll9-1 from *B.mori* genomics. (A) Gene structure of *BmToll9-1*. The number in the bottom indicates the nucleic acids position in the genome sequence. The full length cDNA consists of 8 exons. (B) BmToll9-1 protein structure. BmToll9-1 protein consists of three domains, an ectodomain, a transmembrane domain and a intracytoplasmic domain. (C) Nucleic acid sequence of full length cDNA of BmToll9-1. Start and stop codons are in red font. Signal peptide domain is indicated by dotted underlining. Leucine rich repeats are in blue font. The transmembrane domain is highlighted in yellow. The intracytoplasmic domain is marked by dashed underlining.

In order to confirm the expression and secretion of both the full length protein and ECD of BmToll9-1 in the Bm5 cells, the soluble extracts and insoluble cellular pellets, as well as the culture medium of the transfected cells were examined by Western blot.

Western blot analysis indicated that both BmToll9-1-full and BmToll9-1-ECD were exclusively expressed in Bm5 cellular pellets (Fig. 7.2A). There was no secretion detected in the culture medium despite the presence of signal peptide in both expression constructs.

A similar expression pattern was observed in Hi5 cells for the BmToll9-1-full construct since expression was detected in cellular pellets (Fig. 7.2B). As for BmToll9-1(ECD), expression was observed in the cellular pellets as well as in the soluble cellular extracts (Fig. 7.2B).

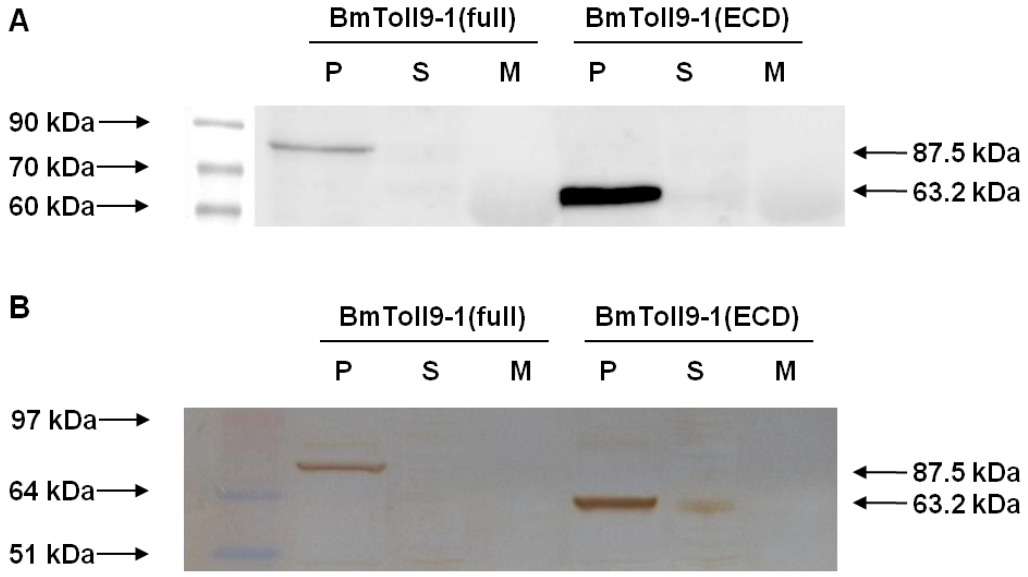


Fig. 7.2. Western blot analysis of expression of BmToll9-1 protein in (A) Bm5 and (B) Hi5 cells. Myc-tagged primary antibody was used to detect the presence of the target protein. Both full protein and ECD of BmToll9-1 accumulated in the pellets of cell extracts. Abbreviations: P, insoluble cellular pellets; S, soluble cellular proteins; M, extracellular medium.

7.3.2. Recombinant BmToll9-1-ECD binds dsRNA

Since BmToll9-1 protein was not expressed in the soluble cellular fractions, it makes it difficult to purify the protein from the insect cell expression system. Thus we switched to the GST fusion expression system to express the ECD of BmToll9-1 (Fig. 7.3A).

As shown in Fig. 7.3A, expression of the target GST fusion proteins was observed only after the addition of IPTG. GST protein (26 kDa) was present in the bacterial soluble and insoluble fractions. GST-Flag-B2 (41 kDa) was also expressed in the soluble and insoluble fractions, while the dominant expression was in the insoluble fraction. GST-BmToll9-1-ECD (87 kDa) was only produced in the insoluble fraction, thus necessitating solubilization before performing purification.

GST-BmToll9-1-ECD could be solubilized using mild detergent (1.5% sarcosyl; (Frangioni and Neel, 1993)). When these samples were applied to Glutathione Sepharose 4B beads, protein binding was observed that allowed purification of GST-BmToll9-1-ECD (Fig.

7.3B). Thus, beads containing GST and its two fusion derivatives were prepared using this method for dsRNA-binding assays.

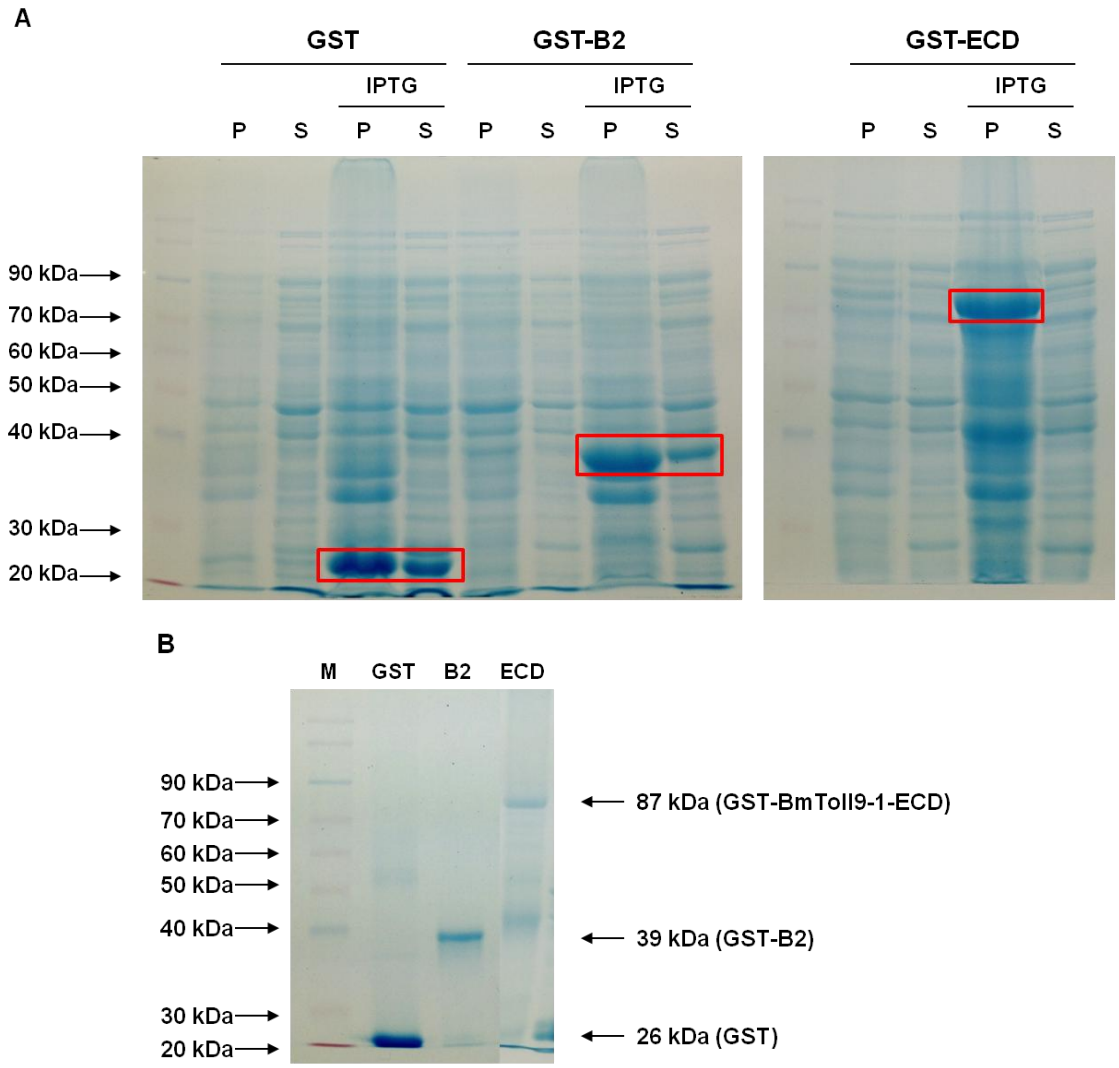


Fig. 7.3. Production and purification of BmToll9-1-ECD GST-fusion protein in bacteria. (A) Coomassie Brilliant Blue-stained 10% SDS-PAGE gel analyzing expression of GST, GST-B2 and GST-ECD proteins in *E. coli* bacteria. Indicated is the induction of expression by IPTG and the type of extracts analyzed (P, bacterial pellets; S, bacterial supernatants). (B) Coomassie Brilliant Blue-stained 10% SDS-PAGE gel showing purified GST, GST-B2 and GST-ECD proteins after binding to Glutathione Sepharose 4B beads.

The beads bound with GST fusion proteins were incubated with dsGFP in binding buffer and then the beads were washed thoroughly with PBS. In this way, only dsGFP that could bind with the proper GST fusion proteins could still bind in the beads. Total RNA was extracted from the beads and RT-PCR was performed to detect binding of dsGFP to the beeads.

GST protein fused to Flag-tagged B2 was used as a positive control because of its documented capacity to bind dsRNA (Ou et al., 2007), while GST was used as a negative

control. According to the RT-PCR (Fig. 7.4), clear amplification of dsGFP was observed in samples which were incubated with Flag-B2, while a weaker signal was obtained for BmToll9-1-ECD, which suggested that BmToll9-1-ECD can bind dsRNA.

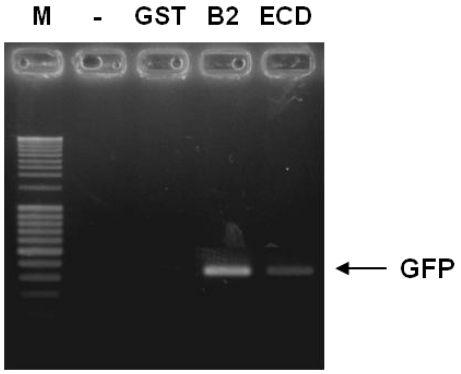


Fig. 7.4. BmToll9-1-ECD dsRNA binding assay. One microgram of dsGFP was incubated with purified GST (20 µg), GST-B2 (3 µg) and GST-BmToll9-1-ECD (1 µg) and washed thoroughly with PBS. Total RNA was extracted and reverse transcribed with dsGFP specific primer. PCR was performed to detect the presence of dsGFP in the samples to indicate the binding of dsGFP.

7.3.3. Absent modulation of RNAi response by BmToll9-1-full in Bm5 cells

In order to investigate whether BmToll9-1 could interfere with the RNAi response, a pERE-Luc-reporter was transfected into Bm5 cells in combination with pEA-BmToll9-1 or pEA-PAC (control), and dsMalE (non-specific dsRNA) or dsLuc (specific dsRNA).

As shown in Fig. 7.5, pEA-PAC was used as a control expression plasmid and the normalized luminescence value in the presence of dsMalE was set for this plasmid at 100%. Substitution of the BmToll9-1 expression plasmid for the PAC expression plasmid resulted in a significant luminescence reduction (75% luminescence compared to pEA-PAC) in the presence of non-specific dsRNA dsMalE. The addition of dsLuc could effectively inhibit luciferase activity in Bm5 cells (Fig. 7.5). Co-transfection of pEA-PAC with dsLuc inhibited the relative luminescence to 33%; while pEA-BmToll9-1-full with dsLuc caused a significant reduction to 21% as well.

When comparing the specific reduction of control expression construct with BmToll9-1 construct, the specific inhibition in the presence of pEA-PAC was 3-fold; while the specific reduction of luminescence in the presence of BmToll9-1 was 3.6-fold (Fig. 7.5). However, statistical analysis indicated that the differences between this two reduction were not significant ($P = 0.29$).

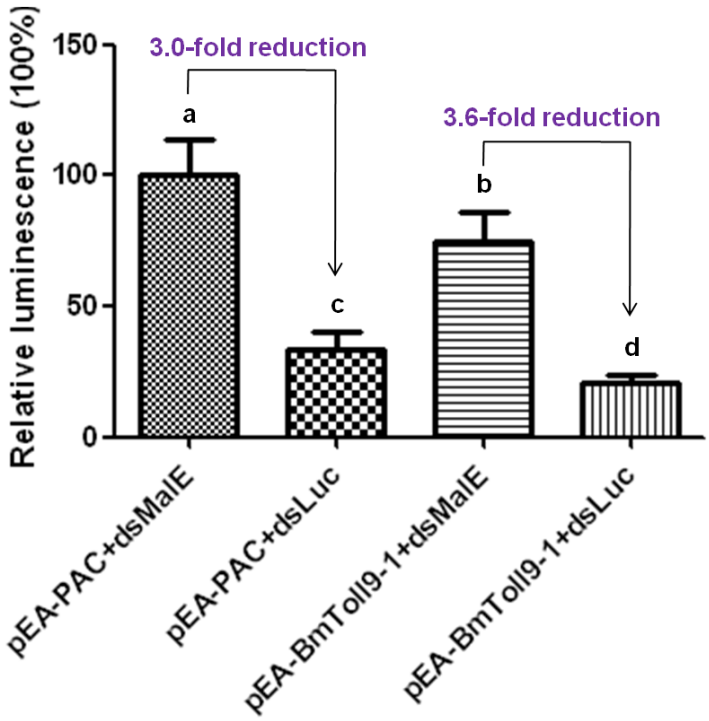


Fig. 7.5. Modulation of the RNAi response by BmToll9-1. Bm5 cells were co-transfected with pEA-PAC (control), pEA-BmToll9-1, dsMalE (non-specific dsRNA) and dsLuc (specific dsRNA). Indicated is the specific reduction in luminescence after transfection with dsLuc.

7.3.4 Confirmation of stable Bm5 transformed cell lines

In order to perform the dsRNA and LPS soaking experiments, two different Bm5 cell lines were generated, one that permanently over-expresses BmToll9-1-full proteins (Bm5-pEA-BmToll9-1) and the other was transformed with the empty expression vector (Bm5-pEA). These two cell lines were selected by puromycin for generations at least one month after transformation.

The different cellular extracts, including soluble extracts and insoluble cellular pellets, as well as culture medium were examined by Western blot. As it indicates in Fig. 7.6, BmToll9-1 protein accumulated in the pellets of the Bm5-pEA-BmToll9-1 cell extracts. No expression was detected in Bm5-pEA control cells, which proves the permanent and stable expression of BmToll9-1 in the transformed cells.

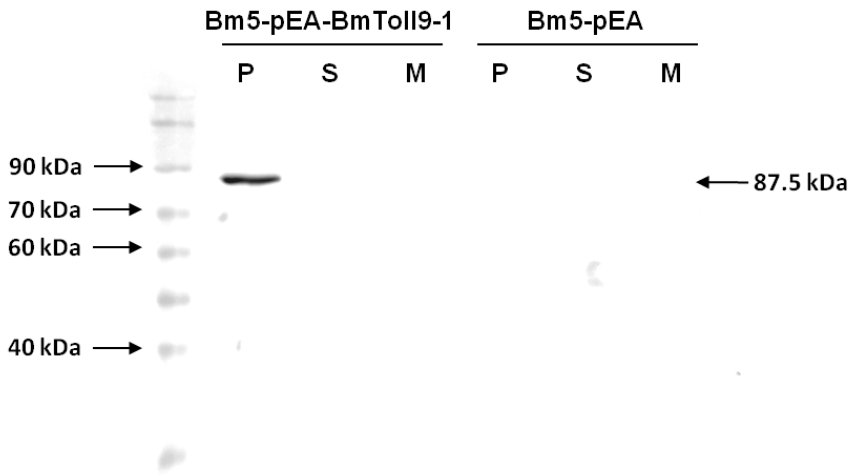


Fig. 7.6. Expression of BmToll9-1 protein in transformed Bm5 cells by Western blot analysis. Abbreviations: P, insoluble cellular pellets; S, soluble cellular proteins; M, extracellular medium.

7.3.5. Expression of innate immune and RNAi-related genes in Bm5 cells

Seven signalling pathway genes of three innate immune response pathways (Toll, IMD, JAK/STAT; Table 2; as described in (Wu et al., 2010b)) were investigated for expression in Bm5 cells and their modulation by Toll9-1 signalling. Regarding RNAi machinery genes, our analysis included genes of the siRNA pathway (Dicer-2, Ago-2) which is involved in the defense against exogenous dsRNA (Siomi and siomi, 2009) as well as the piRNA pathway (Ago-3), of which the involvement in dsRNA-mediated gene silencing in Bm5 cells was recently demonstrated (Kolliopoulou and Swevers, 2013).

Although Bm5 cells are derived from a non-immunogenic tissue (ovary), they were found to be expressing all seven genes of the three immune signalling pathways (Table 7.2). Expression of genes encoding components of the intracellular RNAi machinery (Dicer-2, Ago-2, Ago-3; Table 2) has been reported before (Kolliopoulou and Swevers, 2013; Swevers et al., 2011).

Bm5 cells were also found to be expressing AMP genes, even in the absence of induction, although their expression levels varied significantly. The AMP gene *Defensin* was expressed at the highest levels, which was approximately 10-50-fold higher than *Cecropin E*, *Hemolin* and *Attacin1*. The expression levels of *Lebocin*, *Lysozyme* and *Moricin* were the lowest (approximately 100-500-fold lower than *Defensin*). An overview of the quantification cycle (Cq) values for the detection of the mRNAs of the innate immune, RNAi-related and AMP genes is presented in Table 7.2.

Table 7.2. An overview of the Cq values of the innate immune genes, AMPs and RNAi-related in Bm5 cells.

Gene	Cq			
MyD88	28.74	29.29	27.90	27.60
Rel	29.99	30.16	29.35	29.36
TIF4A	21.36	21.40	20.51	20.58
Imd	28.66	28.81	28.45	28.62
Relish	30.75	30.55	31.20	31.02
Tak1	30.57	30.92	30.27	30.10
TIF4A	20.33	20.71	20.22	20.24
Dome	32.36	31.64	32.24	31.91
Stat	29.91	29.42	29.74	29.74
Att	28.73	28.66	29.14	29.17
TIF4A	20.98	21.06	20.75	20.82
CecE	28.13	27.90	28.53	28.54
Def	24.23	24.10	24.19	24.21
Hem	27.68	27.68	27.83	27.59
TIF4A	20.70	20.89	20.95	20.85
Leb	32.29	31.69	31.59	32.08
Lys	34.48	33.42	33.47	33.66
Mor	33.01	33.53	32.63	32.66
TIF4A	21.24	21.19	20.45	20.64
Dcr2	33.26	33.40	33.82	33.71
Ago2	29.70	29.70	30.55	30.69
Ago3	32.20	32.78	32.20	32.55
TIF4A	21.49	20.96	21.39	21.25

In general, the constitutive expression levels of immune-related genes did not differ much between control cells and cells that express BmToll9-1. Exceptions were *Stat* and *Attacin*, the expression levels of which were reduced to approximately 40% in BmToll9-1-expressing cells (Table 7.3).

Table 7.3. Comparison of constitutive expression between BmToll9-1 line and control cell line. Values are indicated by average and standard deviation. Statistical analysis was compared at least 3 values (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Constitutive expression (Toll:Control)	
Toll pathway:	
MyD88	1.09 ± 0.31
Rel	0.68 ± 0.16 *
IMD pathway:	
Imd	1.41 ± 0.24 *
Relish	0.68 ± 0.29
Tak1	1.06 ± 0.09
JAK/STAT pathway:	
Dome	0.78 ± 0.34
Stat	0.42 ± 0.15 **
AMPs:	
Att	0.42 ± 0.06 **
CecE	1.87 ± 0.66
Def	0.95 ± 0.28
Leb	1.41 ± 0.49
Mor	1.44 ± 0.21 *
Lys	0.74 ± 0.42
Hem	0.78 ± 0.12 **
RNAi pathway:	
Dcr2	0.79 ± 0.27
Ago2	0.88 ± 0.49
Ago3	0.78 ± 0.19

7.3.6. Expression of BmToll9-1 induces the transcription response of innate immune genes in Bm5 cells after dsRNA induction

To determine whether over-expression of BmToll9-1 could affect the transcriptional response of immune genes, expression levels of different innate immune response genes were measured after PAMPs (dsRNA and LPS) administration. Immune genes in the different signaling pathways and AMP genes were analyzed for their transcriptional response by qPCR.

As it shows in Table 7.4, three out of four genes involved in the Toll and JAK/STAT pathways (*Rel*, *Dome* and *Stat*) were significantly induced by dsRNA in BmToll9-1 transformed cells. As for the IMD pathway, only *Relish* was significantly elevated. On the contrary, dsRNA suppressed most genes in the immune signalling pathways in the control cells (Table 7.4). Regarding the AMP genes, dsRNA seems to down-regulate their transcription levels in both cell lines.

Table 7.4. Transcriptional response of innate immune genes and RNAi machinery genes after dsRNA induction in both BmToll9-1 transformed and control Bm5 cells. Comparison was between after and before addition of dsRNA. Values are indicated by average and standard deviation. Statistical analysis was compared at least 3 values (* P < 0.05; ** P < 0.01; *** P < 0.001).Statistic analysis between BmToll9-1 and control cells was also indicated with P value.

	Induction by dsRNA		
	BmToll9-1		Control
Toll pathway:		P = 0.1623	
MyD88	1.48 ± 0.30 *		1.04 ± 0.34
		P = 0.0007	
Rel	2.41 ± 0.45 ***		0.71 ± 0.29
IMD pathway:		P = 0.0082	
Imd	0.88 ± 0.14		1.18 ± 0.05 ***
		P = 0.0039	
Relish	1.90 ± 0.47 **		0.68 ± 0.25 *
		P = 0.6352	
Tak1	0.81 ± 0.21		0.74 ± 0.12 *
JAK/STAT pathway:		P = 0.0010	
Dome	1.85 ± 0.45 **		0.47 ± 0.10 ***
		P = 0.0020	
Stat	3.81 ± 1.32 **		0.36 ± 0.04 ***
AMPs:		P = 0.0264	
Att	1.14 ± 0.23		0.76 ± 0.12 ***
		P = 0.0672	
CecE	0.56 ± 0.26 *		0.89 ± 0.13
		P = 0.0857	
Def	0.60 ± 0.13 ***		0.86 ± 0.23
		P = 0.1154	
Leb	0.91 ± 0.38		0.56 ± 0.12 ***
		P = 0.3674	
Mor	0.75 ± 0.09 **		0.61 ± 0.26 *
		P = 0.0067	
Lys	0.91 ± 0.17		0.44 ± 0.16 ***
		P = 0.2057	
Hem	0.67 ± 0.17 **		0.86 ± 0.20
RNAi pathway:		P = 0.00003	
Dcr2	6.92 ± 0.69 ***		1.86 ± 0.15 ***
		P = 0.0087	
Ago2	1.05 ± 0.35		0.32 ± 0.15 ***
		P = 0.0031	
Ago3	1.41 ± 0.40		0.44 ± 0.07 ***

On the other hand, LPS could induce most of these genes in different signaling pathways in both BmToll9-1 and control cell lines (Table 7.5). However, immune genes in

the IMD pathway, most notably *Tak1*, were more easily induced by LPS in the control cell line (Table 7.5). LPS could also up-regulate the transcription of the AMP genes to a greater extent in the control cell line, which indicates that BmToll9-1 expression diminishes the response to LPS (Table 7.5). Decreased induction by LPS in the BmToll9-1 cell line was most clearly observed for the AMP genes *Attacin*, *Cecropin E*, *Defensin* and *Lysozyme*.

Table 7.5. Transcriptional response of innate immune genes and RNAi machinery genes after LPS induction in both BmToll9-1 transformed and control Bm5 cells. Comparison was between after and before addition of LPS. Values are indicated by average and standard deviation. Statistical analysis was compared at least 3 values (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).Statistic analysis between BmToll9-1 and control cells was also indicated with P value.

	Induction by LPS		
	BmToll9-1		Control
Toll pathway:		P = 0.5513	
MyD88	1.08±0.21		1.21 ± 0.36
		P = 0.0011	
Rel	1.29 ± 0.24 *		0.54 ± 0.10 ***
IMD pathway:		P = 0.0109	
Imd	0.84 ± 0.09 *		1.88 ± 0.56 *
		P = 0.1699	
Relish	1.25 ± 0.21		2.19 ± 1.19
		P = 0.0030	
Tak1	1.49 ± 0.06 ***		3.65± 0.68 ***
JAK/STAT pathway:		P = 0.4025	
Dome	2.89 ± 0.73 **		3.35 ± 0.72 ***
		P = 0.9246	
Stat	1.27 ± 0.15 *		1.28 ± 0.30
AMPs:		P = 0.0012	
Att	3.29 ± 0.29 ***		6.65 ± 1.13 ***
		P = 0.0003	
CecE	1.28 ± 0.43		4.14 ± 0.63 ***
		P = 0.0042	
Def	1.10 ± 0.30		3.37 ± 0.97 **
		P = 0.2639	
Leb	2.16 ± 1.00 *		3.06 ± 1.37 *
		P = 0.0247	
Mor	2.01 ± 0.53 **		4.08 ± 1.29 **
		P = 0.0070	
Lys	1.86 ± 0.90		8.61 ± 2.96 *
		P = 0.0047	
Hem	2.01 ± 0.13 ***		1.39 ± 0.25

Thus, BmToll9-1 expression results in two major changes regarding the immune response in Bm5 cells: (1) it increases the transcriptional response of immune signaling genes to dsRNA; and (2) it decreases the induction of expression of AMP genes in response to LPS.

7.3.7. Expression of BmToll9-1 stimulates the transcription of the RNAi machinery gene Dcr2 in Bm5 cells after dsRNA soaking

Because RNAi is a dsRNA-mediated gene silencing process, only dsRNA was used to treat the cells. When both Bm5 cell lines were soaked with dsRNA, the *Dcr2* mRNA levels were induced particularly in the BmToll9-1 transformed cells (6.92-fold). dsRNA could also up-regulate the transcription of *Dcr2* in control cells but to a significantly lower extent (1.86-fold). On the other hand, it seems that dsRNA had no effect on the transcription of both *Ago2* and *Ago3* in BmToll9-1 cells; while their transcription was significantly suppressed in control cells (Table 7.4).

7.3.8. Immuno-staining of BmToll9-1 proteins indicates localization in the cytoplasm and partial co-localization with lysosomal marker and Flag-tagged Dicer2

In order to confirm the localization of BmToll9-1-full and BmToll9-1-ECD, immuno-staining was performed in transfected Bm5 cells. Wheat germ agglutinin (WGA) staining was used to indicate the cell membrane, while anti-Myc antibodies were used to detect the expression of both Myc-tagged BmToll9-1 constructs. Overlapping of both staining patterns suggested that BmToll9-1-full is exclusively present in the cytoplasm as a diffuse signal (Fig. 7.7A). Staining for BmToll9-1-ECD showed similar localization in the cytoplasm (Fig. 7.7A).

Because mammalian Toll-like receptors are often localized in the endosomal compartment (Alexopoulou et al., 2001), co-staining experiments were carried out with LysoTracker that stains lysosomes, which are part of the endosome transport pathway. In the co-staining experiments, partial overlapping of BmToll9-1-full and LysoTracker was observed even though their overall expression patterns were different. BmToll9-1-full staining generated a diffuse signal while lysosomes appeared as dot-like signals (Fig. 7.7B). A similar partial co-localization pattern with LysoTracker was observed for BmToll9-1-ECD (Fig. 7.7B).

To investigate a possible interaction between BmToll9-1 and the RNAi-machinery, co-staining experiments were also carried out with Flag-tagged Dicer2, the RNase III-type enzyme that processes exogenous dsRNA into siRNA. The staining experiments indicated

partial co-localization of both BmToll9-1 proteins with Flag-tagged Dicer2 in the cytoplasm, although Flag-tagged Dicer2 was also stained more peripherally, close to the cell membrane (Fig. 7.7C). Despite partial co-staining in the cytoplasm, the overall staining pattern of BmTol9-1 was different from Dicer2 (Fig. 7.7C). Nevertheless, this partial co-localization of both BmToll9-1 and Dicer2 proteins in the cytoplasm might indicate that BmToll9-1 could interact with dsRNA that processed by Dicer2, such as binding as we observed in the binding test (Fig. 7.4).

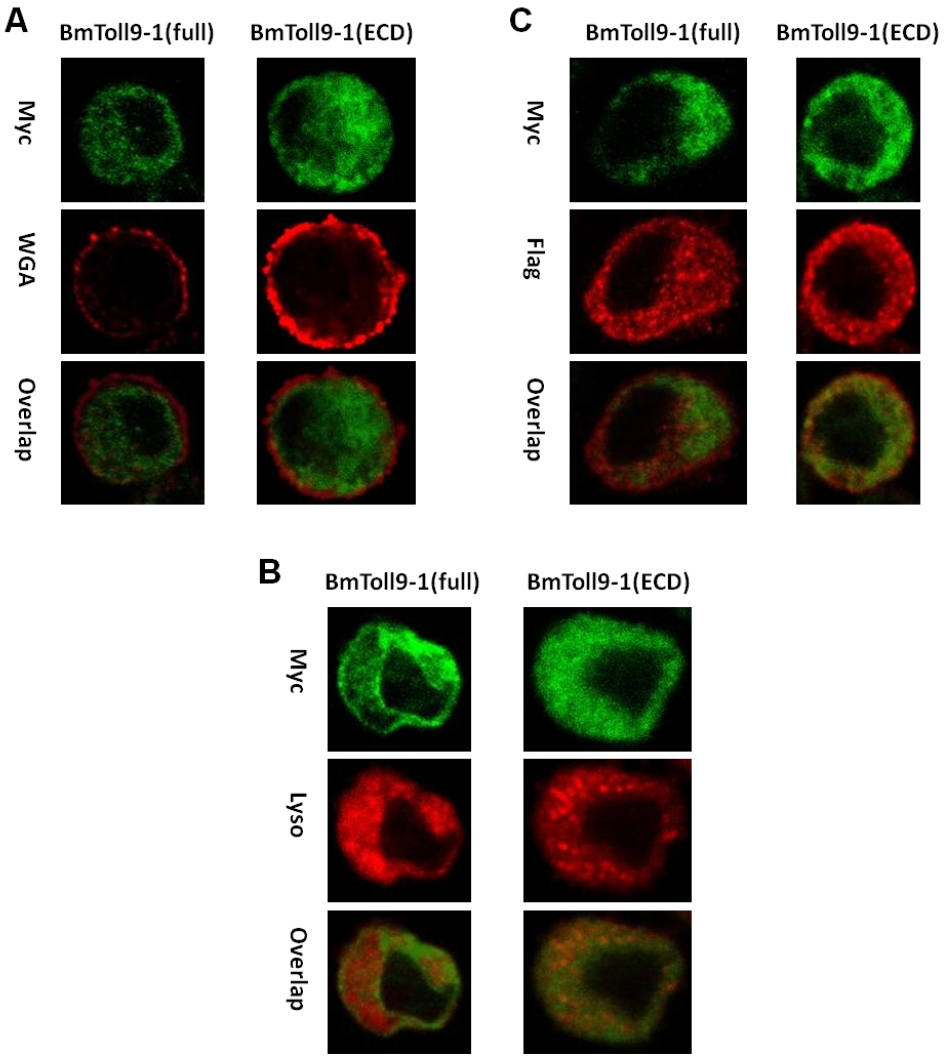


Fig. 7.7. Immunostaining of BmToll9-1-full and BmToll9-1-ECD in Bm5 cells. Shown are co-stainings (A) wheat germ agglutinin (WGA), (B) LysoTracker (Lyso) and (C) Flag-tagged Dcr2 (Flag).

7.4 Discussion

In this chapter, two experimental panels were investigate to study the role of BmToll9-1 in the interaction with dsRNA and the modulation of the immune transduction and RNAi pathways.

To investigate whether dsRNA can interact directly with BmToll9-1, a binding assay was performed with bacterially expressed BmToll9-1-ECD (Fig. 7.4). This assay shows that dsRNA can bind BmToll9-1, which suggests that it may function as a ligand of BmToll9-1 to modulate signaling. Because a non-quantitative approach (RT-PCR) was used, however, other studies need to be carried out, employing, for instance, electrophoretic mobility assays (EMSAs), to determine the affinity and specificity of binding of dsRNA to the ectodomain of BmToll9-1. The result is nevertheless intriguing since phylogenetic analysis showed that BmToll9-1 is more closely related to human TLR3 than to other Toll receptors in *B. mori* (Liu et al., 2013). In mammals, TLR3 functions as a PRR for dsRNA and the interaction TLR3-dsRNA results in the activation of the NF-κB pathway and the production of type-I interferons (Alexopoulou et al., 2001; Doyle et al., 2003).

Because of the observed dsRNA binding property of BmToll9-1, it was also of interest to see whether expression of BmToll9-1 could modulate dsRNA-mediated silencing in Bm5 cells. It was found that expression of BmToll9-1 did not interfere with RNAi of a luciferase reporter (Fig. 7.5). It was also observed that the intracellular localization of BmToll9-1 protein is different from Dicer-2 although partial overlap was observed (Fig. 7.7C). Thus, our data suggest that BmToll9-1 interacts with dsRNA but there is no evidence for BmToll9-1 to modulate directly the intracellular RNAi silencing machinery.

In our previous study, *BmToll9-1* mRNA was found to be strongly expressed in the gut and its expression could be decreased by administration of LPS and dsRNA. This result indicated that *BmToll9-1* suppression constitutes a component of the transcriptional response in the immune reaction against LPS and dsRNA (Liu et al., 2013). Homologs of *BmToll9-1* in other insects were also suggested to play a role in immunity. For example, expression of *Toll9* could trigger a constitutive antimicrobial defense in *D. melanogaster*-derived S2 cells (Ooi et al., 2002). The constitutive activation of AMP genes by DmToll9 in S2 cells was abolished by blocking Toll signaling components, such as MyD88, Pelle and Cactus (Bilak et al., 2003; Ooi et al., 2002), indicating that Toll9 can activate the same signaling pathway as Toll in the antimicrobial defense. On the other hand, *Toll9* mutant flies do not show a defect in the antimicrobial response against fungi and bacteria (Narbonne-Reveau et al., 2011). In addition, normal basal and inducible AMP production was observed in *Toll9* mutants. Despite these observations, it can not be excluded that Toll9 has redundant functions in the antimicrobial immune response (to be uncovered by double mutants). More specifically, it can not be excluded that the Toll9 receptor is important to mediate other aspects of the innate immune response, such as the antiviral response or the response against protozoan parasites

(Narbonne-Reveau et al., 2011). Another report indeed demonstrates that Toll signaling is vital in the antiviral response in *Drosophila* (Zambon et al., 2005). Also in mosquitoes, the Toll signaling pathway has been implicated in the defense reaction against Dengue virus infection (Ramirez and Dimopoulos, 2010; Xi et al., 2008). In *Anopheles gambiae*, *AgToll9* mRNA showed a strong expression in the gut, which might be related to an immune response against pathogens (Christophides et al., 2002).

To investigate in more detail the functional role of the BmToll9-1 receptor in the innate immune response, we took advantage of the observation that silkmoth-derived Bm5 cells do not express this Toll receptor (Liu et al., 2013). Thus, transformed cells that express BmToll9-1 were generated and compared with control cells that underwent the transformation procedure with the empty expression vector.

As it shows in Table 2, both dsRNA and LPS can modulate the transcription of the immune response and AMP effector genes in different immune signaling pathways. These results suggest that both dsRNA and LPS could be recognized as PAMPs to affect the transcriptional response of immune-related genes, which is consistent with our previous experiment (Liu et al., 2013).

dsRNA induced the transcription of most immune signaling genes in the BmToll9-1 transformed cell line. This induction was only observed in the transformed cell line but not in the control cell line (Table 7.4). Meanwhile, *Dcr2* was greatly up-regulated in the transformed cell line compared with the control cell line after dsRNA soaking (Table 7.4). Both results suggest that expression of BmToll9-1 increases the transcriptional response to dsRNA, both at the level of the immune signaling pathway genes and at the level of the RNAi machinery genes.

As for AMP genes, their transcriptional response to dsRNA and LPS is opposite (Table 7.4 & 7.5). AMP mRNA levels were down-regulated by dsRNA but up-regulated by LPS. Although LPS was found to be unable to activate immune-related genes in neither Toll nor IMD pathways in *D. melanogaster* (Kaneko et al., 2004; Leulier et al., 2003), it was reported recently to up-regulate expression of the AMP genes in the fat body of *B. mori* (Tanaka et al., 2009). The activation of immune-related and AMP genes by LPS is therefore confirmed in our experiments.

Although the transcriptional response of AMP genes to dsRNA and LPS is similar in both control and BmToll9-1 cell lines, it is noted that the induction of AMP genes by LPS is diminished in the BmToll9-1 cell line (Table 7.5). This result suggests that BmToll9-1 expression decreases the transcriptional response to LPS. This may be a relevant observation

since Toll9 receptors are mainly expressed in gut tissue (Liu et al., 2013; Luna et al., 2002; Wu et al., 2010a) where there exists a continuous contact with commensal bacteria of the gut content. It is therefore possible that BmToll9-1 is involved in the protection against the premature activation of the immune response by beneficial bacteria in the midgut.

Based on the transcriptional response of the immune genes and the core RNAi genes in the RNAi pathway, it is clear that expression of BmToll9-1 can modulate the innate immune response following dsRNA (and LPS) treatment. Future studies should aim to analyze in more detail the transcriptional response of innate immunity and RNAi pathway genes in the midgut of *Bombyx* (with high expression of BmToll9-1) following LPS and dsRNA administration. So far, only two other reports exist regarding the immune response in the midgut of *Bombyx* (Wu et al., 2010a; Wu et al., 2010b) and these concern infection with bacteria and fungus, but not with dsRNA or virus. Another research avenue would constitute the detailed characterization of the interaction of dsRNA with the ectodomain of BmToll9-1 (together with testing of binding of other ligands) as well as the analysis of the signaling pathway that triggers the transcriptional response of the immune-related genes in transformed Bm5 cells and the midgut.

Chapter 8

General conclusions and future perspectives

Since the discovery of RNAi in the nematode (Fire et al., 1998), RNAi has rapidly developed as a widely used tool in a variety of insect orders, including Diptera (Dietzl et al., 2007; Lum et al., 2003), Lepidoptera (Chen et al., 2008; Terenius et al., 2011; Tian et al., 2009; Yu et al., 2008), Coleoptera (Arakane et al., 2004; Suzuki et al., 2008) and Hymenoptera (Schluns and Crozier, 2007). Because of its high specificity, RNAi provides a reverse genetic technique to study the function of the specific genes and offers great promise on pest control (Baum et al., 2007; Gu and Knipple, 2013; Huvenne and Smagghe, 2010; Price and Gatehouse, 2008).

The first RNAi experiment in insects was successfully reported in *D. melanogaster* (Kennerdell and Carthew, 1998). Afterwards, RNAi became a very popular research tool in functional genomics and the different RNAi pathways are clearly studied in this dipteran model insect (Siomi and Siomi, 2009). In another model insect *T. castaneum*, RNAi is widely used and shows a robust systemic RNAi response (Posnien et al., 2009; Tomoyasu and Denell, 2004). On the contrary, in another model insect in the insect order of Lepidoptera, *B. mori*, the above two RNA silencing pathways are not as clear as in *Drosophila*. A much smaller rate of RNAi efficiency was reported in this insect order, indicating the refractory RNAi efficiency in this insect order (Terenius et al., 2011). Variation in RNAi efficiency exists in Lepidoptera among different species, tissues, stages and target genes (Terenius et al., 2011). This variation might be due to the uptake and delivery of dsRNA, the expression levels of RNAi machinery components and/or presence of RNA degrading enzymes to degrade and clear dsRNA/siRNA/miRNA from the insect body. Therefore, several factors that interact with dsRNA were studied in this doctoral project as shown in Fig. 8.1.

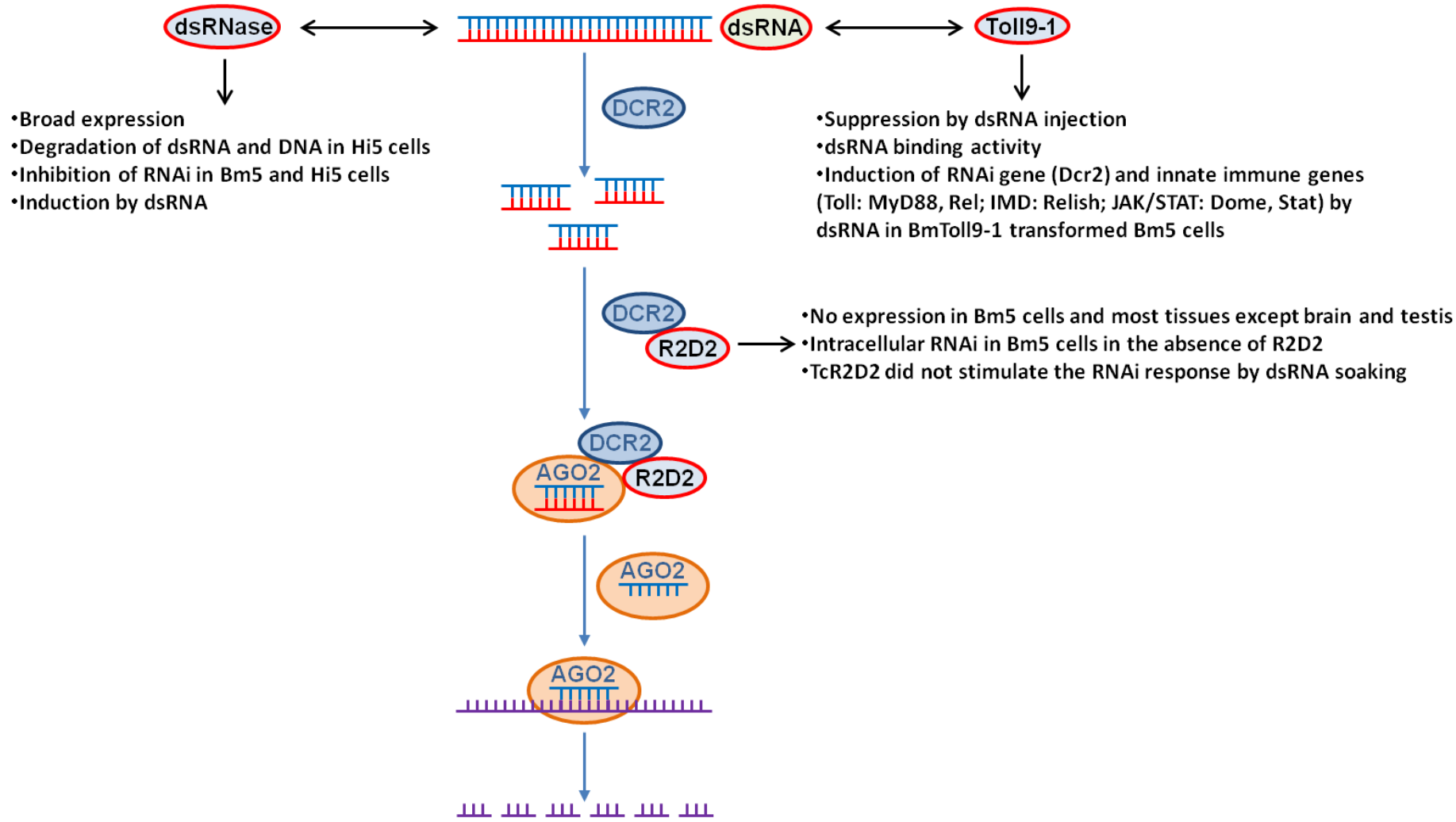


Fig. 8.1. A scheme to summarize the factors that interact with dsRNA in this doctoral project

Table 8.1. Factors studied in this doctoral project and their homologues in various organisms (* indicates the homologues well-studied so far)

Protein	Silkmoth (*Bombyx mori*)	Fruitfly (*Drosophila melanogaster*)	Red flour beetle (*Tribolium castaneum*)	Pea aphid (*Acyrthosiphon pisum*)	Western honey bee (*Apis mellifera*)	African malaria mosquito (*Anopheles gambiae*)	Nematode (*Caenorhabditis elegans*)	Human (*Homo sapiens*)	Mouse (*Mus musculus*)	Plant (*Arabidopsis thaliana*)
R2D2	BmR2D2	DmR2D2*	TcR2D2	ApR2D2	AmR2D2	None	None	None	None	None
dsRNase	BmdsRNase	None	None	None	None	None	None	None	None	None
Toll9-1	BmToll9-1	DmToll9*	None	None	None	AgToll9	None	TLR3*	TLR3*	None

8.1 Broad expression of RNAi pathway machinery genes except R2D2

To gain insight in the mechanism of the RNAi process in lepidopteran insects, we have carried out gene expression studies of the major factors of the siRNA and miRNA pathways in different tissues and at different stages in the silkmoth. In addition, we have focused on the silkmoth-derived Bm5 cell line which is derived from ovarian tissue (Grace, 1967). The core machinery genes in the miRNA pathway: Drosha, Pasha, Dicer-1, Loquacious and Ago-1 (Siomi and Siomi, 2009), and the siRNA pathway: Dicer-2, R2D2 and Ago-2 (Kemp and Imler, 2009) were selected to study the expression patterns.

Expression patterns through RT-PCR showed that almost all the machinery genes involved in the miRNAs silencing pathways have a very broad expression pattern of their mRNAs. Only the mRNA expression of the *Bombyx* Pasha homolog showed clear differences among different tissues, while differences in expression of other factors are generally much less obvious. Similarly, all the machinery genes involved in the siRNAs pathways also have a broad expression pattern except R2D2. To our interest, the expression of BmR2D2 is very low to absent, despite the presence of the gene in the *Bombyx* genome and the annotation of its mRNA in GenBank (accession number NM_001195078). Different sets of primers were designed to amplify BmR2D2, but only a fragment of the 497 bp was generated from brain and gonads of larvae and from testis tissue of pupae after a PCR of 40 cycles, indicating its limited and low expression in the silkmoth.

8.2 Failure of TcR2D2 to stimulate the RNAi response in *Bombyx* cells

Two types of RNAi response in the cells were investigated in the Bm5 cells, depending on the method of dsRNA administration. In case of intracellular RNAi, dsRNA is introduced into the cells with high efficiency by transfection with a lipophilic reagent and the RNAi response can be thought to depend only on the competence of the core RNAi machinery in the cell. In case of extracellular RNAi, dsRNA is supplemented in the growth medium, which is through the endocytosis pathway prior to its presentation to the intracellular RNAi machinery and also defined as "soaking" in the cells.

However, in the absence of BmR2D2 expression, it was observed that intracellular RNAi occurred efficiently in Bm5 cells (Fig. 8.1). Co-transfection of the actin-luciferase reporter with Luc-dsRNA results in a dose-dependent reduction of luciferase activity. In contrast, gene silencing was not detected when the cells were soaked in extracellular medium supplemented with dsRNA.

Because of our unsuccessful attempts to amplify the ORF of *Bombyx* R2D2 (BmR2D2) from tissues of the silkmoth strain that is cultured in our laboratory (Daizo), it was decided to express the R2D2 homolog of *T. castaneum* (TcR2D2; accession NM_001134953). The introduction of an expression construct for TcR2D2 did not influence the potency of luc dsRNA to silence the luciferase reporter (Fig. 8.1). One possible reason is that at the amino acid level, sequence identities between *Bombyx* R2D2 and *Tribolium* R2D2 are only 22%. Thus the possible involvement of BmR2D2 in the RNAi machinery remains to be investigated. In a recent report, partial BmR2D2 gene was isolated and investigated in dsRNA-mediated silencing in Bm5 cells. Blocking of BmR2D2 in Bm5 cells did not cause any inhibitory effect to the dsRNA-mediated silencing. In the mean time, over-expression of BmR2D2 did not stiumuate dsRNA-mediated gene silencing in Bm5 cells. In this report, blocking of another dsRNA-binding protein BmLoqs which involved in the miRNA pathway did not inhibit the RNAi effect cells (Kolliopoulou and Swevers, 2013). These finding could be explained by two alternative mechanisms that may well act in parallel. The first explanation is that there exists another unknown dsRNA-binding protein that can carry out the function of BmR2D2 or BmLoqs in Bm5 cells. The second explanation is that there exists another alternative small RNA silencing pathway that acts in parallel with the siRNA pathway, which requires no dsRNA-binding protein. Hence, BmR2D2 is dispensable for RNAi pathway in Bm5 cells. However, BmR2D2 was localized to cellular membrane when over-expressed in Bm5 cells, it might be expected for Dicer-2/R2D2 complex formation and colocalizes with BmDicer-2, which facilitates the dsRNA soaking in the cells (Kolliopoulou and Swevers, 2013).

8.3 Expression of BmdsRNase results in increased dsRNA degradation in Hi5 cells and inhibition on RNAi response by processed BmdsRNase in Lepidopteran insect cells

dsRNase is grouped in the DNA/RNA non-specific alkaline nucleases since its preferable property to digest dsRNA. While originally reported to be produced by the midgut, it was found that the mRNA of BmdsRNase was also expressed in the epidermis, fat body, gut, muscle, Malpighian tubules, brain, and the silk gland of 5th instar larvae (Fig. 8.1). Because the unexpected broad expression pattern of BmdsRNase, which pointed to additional functions besides its involvement in the digestion of nucleic acids in the food, it was decided to study its functional properties in lepidopteran Hi5 cells, with an emphasis on its possible involvement in the RNAi response.

Our experiments indicate that the isoform BmdsRNase(cat), corresponding to the

mature peptide present in the digestive juice, also exhibited the highest activity when expressed as an intracellular protein (Fig. 8.1). This could be observed in assays that monitor degradation of DNA and dsRNA and dsRNA-mediated gene silencing, where inhibition of the RNAi response was observed (Fig. 8.1) , presumably through degradation of transfected long dsRNAs or processed siRNAs. Immunofluorescence staining of transfected Hi5 cells also showed partial co-localization of BmdsRNase(cat) with Flag-tagged Dicer-2 at asymmetric localizations in the cytoplasm. Of special interest is the capacity of BmdsRNase(full) to degrade dsRNA intracellularly, with possible relevance for the innate immune response against invading nucleic acids with dsRNA structure.

Since purified BmdsRNase from digestive juice could digest dsRNA *in intro*, it would be interesting to test whether dsRNase could cleave dsRNA *in vivo*, which could contribute to explain part of the reasons why RNAi experiments vary in this lepidopteran insects. The detection of dsRNA stability could be performed by qRT-PCR as described in the recent report (Garbutt et al., 2012). On the other hand, our nucleic acids degradation and RNAi response experiments are based on the transient expression of BmdsRNase through transfection of BmdsRNase expression constructs. Therefore, it would also be intriguing to generate a permanent cell line that stably expresses BmdsRNase. With this permanent transformed cell line, dsRNA-mediated gene silening could also be performed to see if over-expression of BmdsRNase could significantly inhibit RNAi response. Meanwhile, dsRNase expressed from the cells could be purified to test its potential to degrade nucleic acids outside the cellular environment.

8.4 Induction of dsRNase and RNAi machinery genes by dsRNA in the larval midgut

Two dsRNA delivery ways, feeding and injection, were applied in the silkmoth larvae. Firstly, to determine the stability of dsRNA, midgut juice and hemolymph were freshly collected from the 5th instar larvae, which corresponding to feeding and injection tests respectively. dsRNA incubated in the midgut juice and hemolymph in vitro suggested that dsRNA was more persistent in the hemolymph, while dsRNA was quickly degraded in the midgut juice in 10 min. Due to the higher persistence of dsRNA in the hemolymph through injection, *dsRNase* was activated and its expression was enhanced after 3 h of dsRNA injection (Fig. 8.1). Similar expression patterns were observed in two RNAi machinery genes, *Dcr2* and *Ago2*, where their expression were also up-regulated by dsRNA injection in 3 h.

8.5 Injection of dsRNA suppresses expression of *BmToll9-1* mRNA in the larval midgut

RNAi is an evolutionary conserved defense response against virus infection or dsRNA structure molecules, but virus or dsRNA could also trigger other pathways in the immune response besides RNAi (Merkling and van Rij, 2012). Toll receptors play a key role in the innate immunity in this process (Takeda and Akira, 2005). While TLRs are the typical PRRs which are able to recognize their specific PAMPs in the immune responses in mammals (Cheng et al. 2008), it remains unknown whether dsRNA could also be recognized as 'foreign' and interact with immunity-related Toll receptors in insects.

An expression pattern study of all 14 Toll receptors in *B. mori* showed that *BmToll9-1* was expressed in different larval and pupal tissues with the highest expression level detected in the midgut, indicating a possible function in immunity against pathogens taken up by the food. In order to investigate the response of *BmToll9-1*, different ways to deliver dsRNA and LPS were applied in *Bombyx* 5^{th} instar larvae. The feeding experiments suggested that dsGFP did not suppress the expression of *BmToll9-1* significantly, while LPS could suppress the expression of *BmToll9-1* after 3h of feeding. On the other hand, the injection experiments showed that dsGFP, as well as LPS, could significantly inhibit the expression of *BmToll9-1* in 3 h (Fig. 8.1). Bacteria that constantly expressed dsGFP could also down-regulate the expression of *BmToll9-1* to a greater extent than bacteria that do not express dsGFP. The failure of dsGFP by feeding to affect the expression of *BmToll9-1* was correlated with the rapid degradation of dsGFP by dsRNase in the midgut juice (Fig. 8.1).

Because LPS is a well-known PAMP, it suggested that the decrease in *BmToll9-1* expression is a consequence of the activation of the innate immune response by LPS. The similar response of *BmToll9-1* between the two triggers, LPS and dsRNA, suggests that dsRNA can also act as a PAMP in the midgut of *Bombyx*. Furthermore, induction of the genes *Dcr2*, *Ago2* and *dsRNase* may also constitute a defense mechanism against invading dsRNA.

8.6 Recombinant BmToll9-1-ECD shows dsRNA binding activity

In order to study the functionality of the BmToll9-1 receptor, the full-length gene was identified from KAIKOBLAST database. Gene structure analysis showed that BmToll9-1 consists of 8 exons and 7 introns. The length of BmToll9-1 cDNA, from start to stop codon, is 2268 bp. Protein structure analysis showed that BmToll9-1 is composed of a signal peptide (21 aa), an LRRs-containing ECD (529 aa), one transmembrane helix (20 aa) and a cytoplasmic TIR domain (185 aa). In order to confirm the expression and secretion of both

the full length protein and ECD of BmToll9-1 in the Bm5 cells, the soluble extracts and insoluble cellular pellets, as well as the culture medium of the transfected cells were examined by Western blot. Western blot analysis indicated that both BmToll9-1-full and BmToll9-1-ECD were exclusively expressed in Bm5 cellular pellets. There was no secretion detected in the culture medium despite the presence of signal peptide in both expression constructs. In the dsRNA-binding assay, the ECD of BmToll9-1 was expressed in the GST fusion expression system. RT-PCR indicated that bacterial expressed recombinant BmToll9-1-ECD could bind dsRNA (Fig. 8.1).

8.7 Induction of innate immune genes and RNAi machinery genes in BmToll9-1 transformed cells

There are different signal transduction pathways involved in the insect innate immune system. These signal transduction pathways include Toll signaling pathway, IMD signaling pathway and JAK/STAT signal pathway. In the immune signaling transduction process, the role of TLRs as PRRs in the innate immunity was determined, very little is known regarding the role of BmToll9-1 in the immune response to mediate other immune pathway genes and RNAi machinery genes.

To investigate the impact of BmToll9-1 on the innate immune genes and RNAi core genes, a transformed Bm5 cell line that permanently expresses BmToll9-1-full was generated and subsequently analyzed for its response to addition of dsRNA or LPS. qRT-PCR suggested that both dsRNA and LPS can be recognized as PAMPs to modulate the transcription of the genes involved in the immune signaling pathways and RNAi pathway (Fig. 8.1). It indicated dsRNA could induced the transcriptional response in most of the immune genes in the immune signaling pathways in the Toll9-1 transformed cell line but not in the control cell line. On the other hand, LPS could up-regulate the transcription of most immune genes and AMPs in both cell lines. Regarding the RNAi core genes, only Dcr2 could be induced by dsRNA especially in the Toll9-1 transformed cell line.

Since BmToll9-1 could bind dsRNA and the transcription response of the immune genes and RNAi core genes induced by dsRNA existed in the BmToll9-1 transformed cell line, it indicates that there should be interaction between BmToll9-1 receptor and dsRNA, suggesting its possible role in immune pathways.

These data indicate that expression of BmToll9-1 can modulate the constitutive expression of RNAi machinery and immune-related genes and their transcriptional response to the PAMPs, LPS and dsRNA. Since bacterially produced BmToll9-1 could bind weakly to

dsRNA in a binding assay, it is possible that dsRNA can alter expression of RNAi- and immune-related genes through direct binding of BmToll9-1. These data contribute to the knowledge of BmToll9-1 to function in the immune signaling pathways and RNAi machinery pathways, which should be taken into account for the design of future RNAi experiments in lepidopteran insects, such as *Bombyx mori*.

Since the studies of Toll receptors in *B. mori* are not so clear as in *D. melanogaster* and mammals, the following questions still need to be answered: Are there any difference between *B. mori* and *D. melanogaster* in the immunity transduction pathways? Could other Toll receptors also influence the innate immune genes in different immune pathways? How do LPS and dsRNA interact with BmToll9-1 to influence other factors and effectors involved in the innate immune pathways?

8.8 Significant findings derived from this project

dsRNA is a hallmark of viral infections, and thus, it is not surprising that the immune system has evolved the capacity to recognize dsRNA and respond to it by mounting antiviral responses. In vertebrates, these innate antiviral responses rely in part on the recognition of dsRNA by TLR3 (Alexopoulou et al., 2001). The consequences of dsRNA recognition include activation of the interferon system, initiation of apoptosis, and inhibition of cellular protein systhesis. On the contrary, dsRNA recognition by RNAi pathway is widely distributed among invertebrates and an important component of the invertebrate antiviral response, which contributes to part of the immune signaling transduction pathways, while the role of dsRNA as a sequence-independent inducer of innate immunity in invertibrates was unrecognized yet.

In this PhD project, several factors that involved in RNAi pathway were selected to study the interaction with dsRNA. First of all, we found that gene specific silencing was achieved in Bm5 cells in the absence of dsRNA-binding factor R2D2, a co-factor for Dicer-2. R2D2 was firstly identified from the *Drosophila* derived S2 cells extracts in 2003 (Liu et al., 2003). Up to date, R2D2 is only identified in insecta, including *Drosophila*, *B. mori*, *Acyrthosiphon pisum*, *Aphis glycines* and *Apis mellifera* (Bansal and Michel, 2013; Tomoyasu et al., 2008), while the functional analysis is mainly on *D. melanogaster* (Liu et al., 2003; Liu et al., 2006).

In the previous report, Dicer-2 and R2D2 could stabilize each other in the insect cells and R2D2 is necessary for Dicer-2 to form the complex to enhance the RNAi efficiency. Dcer-2/R2D2 not only generates siRNA from dsRNA but also binds to siRNA and ficilitates

its loadint onto Ago-2, the central effector in the RISC complex involved in defense against transposable elements and viral infections, indicating that importance and necessarity of R2D2 to bridge the initiation and effector steps of the *Drosophila* RNAi pathway by facilitating siRNA passage from Dicer to RISC (Liu et al., 2003; Liu et al., 2006; Saleh et al., 2006).

Functional analysis of R2D2 on other insects has not been confirmed yet except our research in *Bombyx* (Kolliopoulou and Swevers, 2013; Swevers et al., 2011). Our experiments used R2D2 from *Tribolium* due to the limited expression of R2D2 in the silkmoth tissues and our unsuccess in amplfy the ORF. However, our experiment did not prove that ectopic expression of *Tribolium* R2D2 could stimulate the RNAi effect. In a recent report, blocking of BmR2D2 in Bm5 cells did not cause any inhibitory effect to the dsRNA-mediated silencing. In the mean time, over-expression of BmR2D2 did not stiumuate dsRNA-mediated gene silencing in Bm5 cells (Kolliopoulou and Swevers, 2013). However, in this report, they only used partical R2D2 to perform the functional anaysis instead of the ORF of R2D2. Hence, the involvement of R2D2 in *Bombyx* has not been really confirmed yet. Since the formal demonstration that RNAi in Lepidoptera is working through the generation of siRNAs by a Dicer-2/R2D2 complex has not been published yet, it would also be valuable to isolate full-length BmR2D2 from appropriate silkmoth strains and test whether BmDicer-2/BmR2D2 dimers can generate siRNAs and load them to RISC as is observed for the DmDicer-2/DmR2D2 complex in purified S2 cell extracts (Liu et al., 2006).

The second factor that we studied is dsRNase, a DNA/RNA non-specific alkaline nuclease that has the interesting property to digest dsRNA and is therefore sometimes designated as dsRNase. This enzyme was first detected in the digestive juice and midgut tissues (Mukai, 1965) and was characterized as a Mg^{2+}-dependent alkaline nuclease degrading both DNA and dsRNA (Arimatsu et al., 2007a). This enzyme is present in Lepidoptera only, based on the database available nowadays, not even in other insects such as *Drosophila* or *Tribolium*. Hence, our functional analysis of this enzyme in the involvement in dsRNA cleavage and RNAi response is quite novel. Our experiments proved that mature form of dsRNase could function in dsRNA degradation and RNAi inhibition. Since this enzyme is uniquely adapted to digest nucleic acids in the alkaline environment of the midgut of Lepidoptera and its borad expression pattern in *B. mori*, it might explain the refractoriness of RNAi in this order.

The third factor we studied is Toll9-1, a homolog receptor of DmToll9 and relatively close to mammalian TLR3. In *Drosophia* and mammals, experiments on this receptor focus

on immune response only. However, we studied this receptor not only on immune response but also on RNAi response. Although we did not prove that BmToll9-1 could significantly influence RNAi response in Bm5 cells, we confirmed that BmToll9-1 could bind dsRNA, which was only known in mammals but not in other insects. Our cell soaking with dsRNA and LPS assay proved that BmToll9-1 could interact with dsRNA to induce the transcriptional response of the immune genes involved in different immune signaling pathways. In the mean time, LPS could up-regulate the transcriptional response of most immune genes and AMPs, indicating its presence of immune transduction pathways in *B. mori*. Therefore, dsRNA might also interact with these immune genes in *B. mori*, instead of participating in dsRNA-mediating gene silencing pathway, which also contributes to the variation of RNAi efficiency in Lepidoptera.

8.9 Open questions: Can immune response to dsRNA explain the low efficiency of RNAi efficiency in lepidopteran insects?

Many insights into innate immunity have been obtained from studies in *Drosophila*. Moreover, many of the classic signal transduction systems, including those involved in immunity, were identified first in *Drosophila* using forward genetic screens (Lemaitre et al., 1996). *Drosophia* clearly respond to viral infections by initiating an antiviral program that includes the small RNA silencing pathways, and a number of signaling cascades that converge on effector mechanisms including autophagy (Ding, 2010; Merkling and van Rij, 2012). The immune signaling pathways in *Bombyx* and other lepidopteran insects are not extensively studied like in *Drosophila*. However, the entire genome analysis of *B. mori* was almost completed (Consortium, 2008), and a subsequent genome-wide analysis of immune-related genes was performed (Tanaka et al., 2008). this analysis revealed that the factors involved in intracellular signal transduction pathways are well conserved between silkworm and non-lepidopteran insects, whereas the recognition proteins and effectors are structurally diverse among them. As for genes encoding recognition proteins and effectors, a dynamic lineage-specific gene evolution probably occurred in Lepidoptera to adapt the silkmoth or lepidopteran insects to the pathogens that preferentially infect them, e.g., *Moricin, Lebocin, Gloverin* and *Hemolin* are immune genes that have evolved specifically in lepidopterans (Tanaka et al., 2008). Unfortunately, the function of most genes encoding recognition proteins in *B. mori* is unknown.

Nevertheless, we took advantage of the model lepidopteran insect *B. mori* and its ovary-derived Bm5 celll line that is easy to produce exogenous recombinant proteins, in

combination of RNAi-based luciferase silencing system, to study the interaction among dsRNA, RNAi core gene, dsRNA-degrading enzyme and immune genes.

First, our interested to investigate whether RNAi core gnes and dsRNA-degrading enzymed are up-regulated in response to dsRNA in insects, which derived from the variation of RNAi efficiency in lepidopteran RNAi experiments (Terenius et al., 2011). Our experiments reveailed that both RNAi core genes, Dcr2 and Ago2, as well as the dsRNA-degrading enzyme, dsRNase, are up-regulated by dsRNA, which is consistent with the similar reports, where they found that Dcr2 and Ago2 was induced by dsRNA in *M. sexta* larval tissues but not other RNAi machinery components (Garbutt and Reynolds, 2012). The induction of Dcr2 and Ago2 genes by dsRNA but not other RNAi machinery components might due to their roles in addition to function in processing intracellular dsRNAs in RNAi pathway, for example as immune PPRs. Evidence for a role of Dcr2 as a viral PRR was provided in a report, where they found the induction of *D. malanogaster* gene *vago*, one of the genes with highest induction upon DCV infection, was depedent on Dcr2 (Fig. 2.4) (Deddouche et al., 2008). Meanwhile, dsRNA is responsive to dsRNA induction, which might also act as a PRR in immune response, which was proved in another report that dsRNase has been hypothesized to function in the innate immune response against *Bombyx* CPV, a virus with dsRNA genome structure (Wu et al., 2009). Such defense function would require intracellular expression of the enzyme where it could attack exposed replication intermediates efficiently. The RNAi machinery, consisting of Argonaute 2, Dicer-2 and R2D2, has also been implicated in antiviral defense (Saleh et al., 2009) and dsRNase activity could contribute to the further degradation of cleaved products from the RISC complexes, which might contribute to the low RNAi efficiency in Lepidoptera.

Second, potent AMPs induction, effector of immune respons, have only been reported in some virus infections of *Drosophila* and mosquito, but not dsRNA, while immune induction by dsRNA is recognized by TLR3 in mammals, resulting in activation of the interferon system, initiation of apoptosis, and inhibition of cellular protein systhesis (Alexopoulou et al., 2001; Merkling and van Rij, 2012). In our experiments, dsRNA did not trigger the AMPs induction in Bm5 cells, while LPS could induce the transcription of AMPs significantly. Since LPS is a typical PAMP in mammals (Takeda and Akira, 2004, 2005), it is not surprising that LPS can trigger the transcription of AMPs. Although dsRNA did not induce the transcription of AMPs in our experiments, it induced the transcription of most of the immune genes in Toll, IMD and JAK/STAT pathways in the presence of Toll9-1. Since Toll9-1 is dominantly expressed in *Bombyx* larvae, this induction of immune genes by

dsRNA could not be excluded in larvae which are applied with RNAi experiments. Hence, dsRNA might also interact with these immune genes instead of participating in dsRNA-mediating gene silencing pathway.

However, the downsteam immune effectors for Toll, IMD and JAK/STAT pathways still remain to be identified if dsRNA is involved. It is still unclear whether AMPs directly contribute to antivral defense in *Drosophila* and Lepidoptera even though AMPs seem to participate in antiviral responses in mosquitoes (Merkling and van Rij, 2012). Hence, even our experiments point out that dsRNA might be recognized as a PAMP to trigger the response of potent PRRs involved in RNAi machinery components, such as Dcr2, Ago2, dsRNase and Toll9-1, which might explain this variation of RNAi efficiency in Lepidoptera. The adaption proteins and effectors in these pathways still need to be clarified. Nevertheless, this project still contributes to our current knowledge of RNAi machinery pathways and the immune signaling pathways, which should be taken into account for the design of future RNAi experiments in lepidopteran insects, such as *B. mori*.

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Summary

RNA interference (RNAi) is a mechanism of post-transcriptional regulation of gene expression. It is a process where exogenous double-stranded RNA (dsRNA) silences the complementary endogenous messenger RNA (mRNA). Since being discovered in the nematode *Caenorhabditis elegans*, RNAi has rapidly developed as a widely used functional genomics tool in a variety of insect orders. The RNAi pathways are extensively studied in the dipteran model insect *Drosophila melanogaster* and a robust systemic RNAi response is shown in another coleopteran model insect *Tribolium castaneum*. However, the RNAi pathways are still not clear and a smaller rate of efficiency was reported in Lepidoptera, including the model insect *Bombyx mori*. This refractoriness might be due to the uptake and delivery of dsRNA, the expression levels of RNAi machinery components and/or presence of RNA degrading enzymes to degrade and clear dsRNA/siRNA/miRNA. Therefore in this doctoral project, several factors, including R2D2, dsRNase and Toll9-1, that interact with dsRNA were studied in the silkmoth *B. mori* and lepidopteran cell lines.

To gain insight into the factors that determine the efficiency of RNAi, in Chapter 3, a survey was carried out to check the expression of factors that constitute the small interfering RNA (siRNA) and microRNA (miRNA) machinery pathways in different tissues and stages of the silkmoth, *B. mori*. It was found that all the factors showed a broad expression pattern except the dsRNA-binding protein R2D2, an essential component in the siRNA pathway in *Drosophila*.

To our interest, the expression of BmR2D2 is very low to absent, despite the presence of the gene in the *Bombyx* genome and the annotation of its mRNA in GenBank. The silkmoth-derived Bm5 cell line was also deficient in the expression of R2D2. To study its possible involvement in the RNAi efficiency, two types of RNAi (intracellular and extracellular RNAi) were investigated in Chapter 4. Despite the lack of expression of R2D2, it was observed that intracellular RNAi occured efficiently in Bm5 cells. Co-transfection of the actin-luciferase reporter with Luc-dsRNA resulted in a dose-dependent reduction of luciferase activity. Due to our unsuccessful attempts to amplify the ORF of *Bombyx* R2D2 (BmR2D2), the R2D2 homolog from *T. castaneum* (TcR2D2) was cloned for the subsequent studies. The introduction of an expression construct for TcR2D2 did not influence the potency of luc dsRNA to silence the luciferase reporter. In the meantime, ectopic expression of TcR2D2 did not result in silencing of luciferase activity following addition of Luc-dsRNA to the culture medium. One possible reason is that at the amino acid level, sequence identities between *Bombyx* R2D2 and *Tribolium* are only 22%. Thus the possible involvement of BmR2D2 in the RNAi machinery remains to be investigated.

In Chapter 5, a DNA/RNA non-specific alkaline nuclease (BmdsRNase) was cloned to study its functional properties in dsRNA degradation and RNAi efficiency. While originally reported to be produced by the midgut only, it was found that the mRNA of this enzyme was also expressed in the epidermis, fat body, gut, muscles, Malpighian tubules, brain, and silk glands of 5th instar larvae, indicating additional functions to its reported role in nucleic acid digestion in the midgut. Different pEA-BmdsRNase expression constructs were generated, characterized by the presence or absence of a signal peptide and a propeptide. These expression constructs were used for expression in lepidopteran Hi5 tissue culture cells. Western blot indicated that these different forms of BmdsRNase protein were not secreted into the growth medium, while they were detected in the pellets and supernatants of Hi5 cell extracts. Nucleic acids cleavage experiments indicated that full-length of BmdsRNase could digest dsRNA and that the processed form (absence of signal peptide and propeptide) of BmdsRNase could degrade both DNA and dsRNA in Hi5 cell culture. Using a reporter assay targeted by transfected homologous dsRNA, it was shown that the digestive property of the processed form could interfere with the RNAi response. Immunostaining of processed BmdsRNase protein showed asymmetric localization in the cellular cytoplasm and co-localization with Flag-tagged Dicer-2 was also observed. These results indicated that intracellular protein isoforms of BmdsRNase can be functional and involved in the regulation of nucleic acid metabolism in the cytoplasm. In particular, because of its potency to degrade dsRNA, the enzyme might be involved in the innate immune response against invading nucleic acids such as RNA viruses.

In RNAi experiments, injection of dsRNA is widely applied to silence endogenous genes and study gene function in insects. However, it is not yet clear to what extent it can also exert non-specific effects, for instance by interference with the innate immune response. In Chapter 6, an immune gene BmToll9-1 was selected to study its transcriptional response to exogenous dsRNA in the midgut of *B. mori*. In order to investigate the response of *BmToll9-1* against dsRNA, different ways to deliver dsRNA (dsGFP) were applied in *Bombyx* 5th instar larvae. Since lipopolysaccharide (LPS) is a well-known pathogen-associated molecular pattern (PAMP), LPS was tested in parallel. Due to the rapid degradation of dsRNA by dsRNase in the midgut juice, dsRNA feeding experiments did not affect the expression of *BmToll9-1*. Instead, dsRNA was more persistent in the hemolymph and the expression of *BmToll9-1* was significantly inhibited by dsRNA injection. Feeding and injection with LPS could also suppress the expression of *BmToll9-1*. The similar response of *BmToll9-1* between the two triggers, LPS and dsRNA, suggests that dsRNA can also act as a PAMP in the midgut

of *Bombyx*. In the meantime, two RNAi machinery components, including *Dcr2* and *Ago2*, as well as *dsRNase*, were also studied for their transcription response against dsRNA. Expression of *Dcr2*, *Ago2* and *dsRNase* was also affected by injection of dsRNA and not by feeding, but in these cases an increase was observed instead of a down-regulation, which might constitute a defense mechanism against invading dsRNA.

In Chapter 7, to investigate the impact of BmToll9-1 on the innate immune and RNAi core genes, in this project, we generated a transformed Bm5 cell line expressing permanently BmToll9-1 (full and ECD separately), and subsequently analyzed for binding of dsRNA and transcriptional responses to exposure of LPS and dsRNA. Major results were, in first, that recombinant BmToll9-1 could bind dsRNA, and in second, both dsRNA and LPS were recognized as PAMPs. dsRNA induced the transcriptional response in most of the genes of the innate immune pathway in the Toll9-1 expressing cell line, but not in the control cell line. In contrast, LPS could up-regulate the transcription of most innate immune genes and AMPs in both cell lines. Regarding the RNAi core genes, only *Dcr2* could be induced by dsRNA especially in the Toll9-1 expressing cell line. In addition, under the confocal microscope, we observed that Toll9-1 (full and ECD) was exclusively expressed in the cytoplasm and partially co-stained with Flag-tagged Dicer2, the RNase III-type enzyme that processes exogenous dsRNA. Since BmToll9-1 could bind dsRNA and the transcription response of the immune genes and RNAi core genes induced by dsRNA existed in the BmToll9-1 transformed cell line, it indicates that there should be interaction between BmToll9-1 receptor and dsRNA, suggesting its possible role in immune pathways.

In conclusion, three fatcors were studied in this doctoral project to investigate their possible interaction with dsRNA. Experiments regarding the first factor, dsRNA-binding factor R2D2, indicated that gene specific silencing was achieved in Bm5 cells in the absence of R2D2. However, ectopic expression of TcR2D2 did not stimulate the RNAi response in Bm5 cells. Thus it is valuable to isolate full-length BmR2D2 from appropriate silkmoth strains and test whether BmDicer-2/BmR2D2 dimers can generate siRNAs and load them to RISC to interfere with the RNAi response. Experiments on the second factor dsRNase showed that the catalytic form of dsRNase could degrade both dsRNA and DNA in Hi5 cells and inhibit the RNAi response in lepidopteran Hi5 and Bm5 cells. As for the third factor BmToll9-1, recombinant BmToll9-1-ECD could bind dsRNA, it only triggered minor RNAi modulation in Bm5 cells, indicating no direct interaction between BmToll9-1 and the RNAi machinery. Nevertheless, dsRNA can act as a PAMP to down-regulate the mRNA transcription of *BmToll9-1* in the larval midgut tissues, which instead up-regulated the

transcripiton of *Dcr2*, *Ago2* and *dsRNase*. Soaking the transformed BmToll9-1 Bm5 cell line with dsRNA showed an enhanced transcriptional response of most immune genes involved in different immune transduction pathways and *Dcr2* in RNAi pathway. All the data contribute to our current knowledge on the RNAi machinery and the interaction with immune signaling pathways, which should be taken into account for the design of future RNAi experiments in lepidopteran insects, such as *B. mori*.

Samenvatting

RNA interferentie (RNAi) is een post-transcriptioneel genexpressie regulatie mechanisme. Het is een proces waarbij exogeen dubbelstrengig RNA (dsRNA) in staat is afbraak van het complementaire endogene boodschapper RNA (mRNA) te veroorzaken. Sinds de ontdekking van RNAi in de nematode *Caenorhabditis elegans* is de techniek snel uitgegroeid tot een veelgebruikte onderzoekstechniek in insecten. De RNAi pathways zijn in detail bestudeerd bij het dipteer modelorganisme *Drosophila melanogaster* en een robuuste systemische RNAi respons werd aangetoond bij het coleopteer modelorganisme *Tribolium castaneum*. Desondanks is de precieze werking van de RNAi pathway nog steeds niet helemaal opgehelderd. In de orde van de Lepidoptera, waartoe het model insect *Bombyx mori behoort*, wordt een lagere efficiëntie voor RNAi geobserveerd. Deze gebrekkige efficiëntie kan mogelijks te maken hebben met de toediening en opname van dsRNA, de expressieniveaus van elementen van de RNAi machinerie en/of de aanwezigheid van RNA degraderende enzymen die dsRNA/siRNA/miRNA kunnen afbreken. Daarom werden in dit doctoraatsproject een aantal factoren die deze efficiëntie kunnen beïnvloeden, waaronder R2D2, dsRNases en Toll9-1, onderzocht in de zijderups *B. mori* en lepidopteer-cellijnen.

Om een verder inzicht te bekomen in de factoren die RNAi efficiëntie bepalen werd in Hoofdstuk 3 onderzoek gedaan naar de expressie van een aantal genen die deel uitmaken van de small interfering RNA (siRNA) en microRNA (miRNA) pathways in verschillende weefsels en levensstadia van de zijderups *B. mori*. We vonden dat alle onderzochte RNAi machinerie elementen duidelijk tot expressie kwamen behalve het dsRNA-bindings eiwit R2D2, een essentiële component in de siRNA pathway in *Drosophila*.

De expressie van *BmR2D2* is heel laag tot afwezig, ook al is het gen aanwezig in het genoom van *Bombyx* en is het mRNA geannoteerd in GenBank. Ook in de Bm5 cellijn, afkomstig van de zijderups, bleek *R2D2* niet tot expressie te komen. Om de invloed hiervan op de lage RNAi efficientie te onderzoeken werden twee types van RNAi (intracellulair en extracellulair RNAi) onderzocht in Hoofdstuk 4. Ondanks de afwezigheid van *R2D2* expressie vonden we dat intracellulair RNAi efficiënt werkte bij Bm5 cellen. Co-transfectie van het actine-luciferase reporter construct met Luc-dsRNA resulteerde in een dosis-afhankelijke vermindering van luciferase activiteit. Doordat we er niet in slaagden het ORF van *Bombyx* R2D2 (BmR2D2) te amplificeren werd het R2D2 homoloog van *T. castaneum* (TcR2D2) gekloneerd voor onze verdere studies. De introductie van een expressieconstruct voor TcR2D2 had geen invloed op de silencing van de luciferase reporter door luc-dsRNA. Ectopische expressie van TcR2D2 resulteerde ook niet in silencing van de luciferase activiteit wanneer Luc-dsRNA aan het cultuurmedium werd toegevoegd. Eén mogelijke verklaring is

dat de aminozuur-sequenties van *Bombyx* en *Tribolium* R2D2 slechts 22% identiteit vertonen. De mogelijke betrokkenheid van BmR2D2 in de RNAi machinerie moet bijgevolg nog verder onderzocht worden.

In Hoofdstuk 5 werd een DNA/RNA niet-specifiek alkaline nuclease (BmdsRNase) gekloneerd om z'n functionaliteit bij dsRNA degradatie en betrokkenheid bij RNAi efficiëntie te bestuderen. Hoewel eerder gedacht werd dat het enzym enkel in de middendarm van de zijderups voorkwam, hebben we aangetoond dat het ook in de epidermis, het vetlichaam, de darm, spierweefsel, buizen van Malpighi, hersenen en zijdeklieren van het 5de larvaal stadium tot expressie komt. Dit toont aan dat het nog andere functies kan hebben naast de eerder gerapporteerde dsRNA-vertering in de darm. Verschillende pEA-BmdsRNase expressie constructen, gekarakteriseerd door de aan- of afwezigheid van een signaalpeptide en een propeptide, werden aangemaakt. Deze constructen werden vervolgens tot expressie gebracht in Hi5 celculturen. Western Blot analyse toonde aan dat deze verschillende vormen van het BmdsRNase eiwit niet werden gesecreteerd in het cultuurmedium, terwijl ze wel werden gedetecteerd in pellet en supernatans van de extracten van deze Hi5 cellen. Nucleïnezuur-degradatie experimenten toonden aan dat het volledige BmdsRNase eiwit in staat is het dsRNA te degraderen en dat de gemodificeerde vorm zowel DNA als dsRNA kon degraderen in Hi5 cellen. Aan de hand van een reporter assay kon aangetoond worden dat de degradatie-eigenschappen van de gemodificeerde vorm de RNAi respons kon verstoren. Immunokleuring van het gemodificeerde BmdsRNase eiwit bracht een asymmetrische lokalisatie in het cellulair cytoplasma aan het licht en co-lokalisatie met Flag-gebonden Dicer-2 werd ook opgemerkt. Deze resultaten toonden aan dat intracellulaire isovormen van BmdsRNase functioneel kunnen zijn en betrokken in de regulatie van nucleïnezuren zoals RNA virussen.

RNAi via injectie van dsRNA is een veelgebruikte tool bij insecten om genen uit te schakelen en zo hun functie te onderzoeken. Het is echter nog niet duidelijk in welke mate niet-specifieke effecten kunnen veroorzaakt worden, bijvoorbeeld door interactie met de natuurlijke immuunrespons. In Hoofdstuk 6 werd een gen dat betrokken is bij die immuunrespons, namelijk *BmToll9-1*, geselecteerd om de transcriptionele respons te onderzoeken na toediening van exogeen dsRNA in de middendarm van *B. mori*. *BmToll9-1* codeert voor een Toll receptor die een hoge expressie vertoont in middendarm weefsel en een beperkte gelijkenis gevonden met TLR3 bij zoogdieren, een endosoom receptor voor dsRNA. Om de respons van *BmToll9-1* op dsRNA te onderzoeken werd dsRNA (dsGFP) via

verschillende methoden toegediend aan het 5de larvaal stadium van *Bombyx*. Aangezien LPS een bekende PAMP is, werd deze in parallel getest.

De voedingsexperimenten suggereerden dat dsGFP de expressie van *BmToll9-1* niet significant onderdrukte, terwijl LPS wel in staat was om de expressie van *BmToll9-1* te onderdrukken, na 3 uur voeden. Bij de injectie-experimenten echter werd zowel voor dsGFP als voor LPS een duidelijke inhibitie van *BmToll9-1* expressie aangetoond binnen de drie uur na toediening. Bacteriën die dsGFP continu tot expressie brengen resulteerden ook in een downregulatie van de *BmToll9-1* expressie in grotere mate dan bacteriën die geen dsGFP tot expressie brachten. De afwezigheid van een effect op de *BmToll9-1* expressie na toedienen van dsGFP via de voeding was gecorreleerd met de snelle degradatie van dsGFP door dsRNase in de darmsappen. De expressie-respons van componenten van de RNAi machinerie, waaronder Dcr2 en Ago, alsook het dsRNase, na toediening van dsRNA werd ook bestudeerd. Expressie van deze drie genen werd duidelijk beïnvloed door injectie van dsRNA maar niet bij het toedienen via de voeding. In deze gevallen werd echter een verhoogde expressie geobserveerd in plaats van een onderdrukte expressie. De gelijkaardige respons van BmToll9-1 bij de twee triggers, LPS en dsRNA, wijst uit dat dsRNA ook kan fungeren als een PAMP in de middendarm van *Bombyx*. Bovendien kan de verhoogde expressie van Dcr2, Ago2 en dsRNase ook wijzen op een verdedigingsmechanisme tegen binnendringend dsRNA.

Om de impact van BmToll9-1 op de aangeboren immuniteit en RNAi core genen te testen werd een getransformeerde Bm5 cellijn aangemaakt die continu BmToll9-1 tot expressie bracht (volledig en ECD afzonderlijk). Vervolgens werd bij deze cellijn de binding van dsRNA en de transcriptionele respons op blootstelling aan LPS en dsRNA onderzocht. Daaruit bleek ten eerste, dat recombinant BmToll9-1 dsRNA kon binden en, ten tweede, dat zowel dsRNA als LPS herkend werden als PAMPs. In de getransformeerde cellijn induceerde dsRNA de transcriptionele respons in het grootste deel van de genen behorend tot deze aangeboren immuniteit pathway. Dit was echter niet het geval in de controle cellijn. Blootstelling aan LPS echter, veroorzaakte een opregulering van de meeste van deze genen in beide cellijnen. Van de RNAi core genen kon enkel *Dcr2* geïnduceerd worden door dsRNA, vooral in de cellen die Toll9-1 tot expressie brengen. Daarenboven observeerden we via confocale microscopie dat Toll9-1 enkel voorkwam in het cytoplasma van de cellen en gedeeltelijk associeerde met Flag-tagged Dicer2, het RNase III-type enzyme dat exogeen dsRNA knipt. Het feit dat BmToll9-1 kan binden met het dsRNA en een transcritptionele respons van the immuniteitsgenen en RNAi core genen, geïnduceerd door dsRNA, werd

geobserveerd in de BmToll9-1 getransformeerde cellijn, indiceert dat er een interactie tussen de BmToll9-1 receptor en dsRNA plaatsvindt, wat wijst op een mogelijke rol in de immuunpathways.

Uiteindelijk werden drie factoren bestudeerd in dit doctoraatsproject die mogelijks kunnen interageren met dsRNA. Experimenten die de eerste factor, het dsRNA bindend eiwit R2D2, onderzochten toonden aan dat gen-specifieke silencing mogelijk was in Bm5 cellen zonder de aanwezigheid van R2D2. Bovendien stimuleerde ectopische expressie van TcR2D2 de RNAi respons in Bm5 cellen niet. Het zou dus waardevol kunnen zijn om het complete BmR2D2 gen uit de geschikte zijderups-stammen te isoleren en om vervolgens te onderzoeken of BmDicer-2/BmR2D2 dimeren siRNAs kunnen genereren en ter beschikking stellen van het RISC om zo een RNAi-respons te genereren. Experimenten betreffende de tweede factor, het dsRNase, toonden dat de katalytische vorm van het dsRNase zowel dsRNA als DNA kon degraderen in Hi5 cellen en de RNAi respons in lepidopteren cellijnen Hi5 en Bm5 konden inhiberen. Wat de derde factor betreft, hoewel recombinant BmToll9-1-ECD kon binden met dsRNA, zorgde het enkel voor een kleine RNAi modulatie in Bm5 cellen, wat erop wijst dat er geen directe interactie is tussen BmToll9-1 en de RNAi machinerie. Desondanks kan dsRNA wel acteren als PAMP om de mRNA transcriptie van *BmToll9-1* te inhiberen in de larvale middendarm weefsels. Dit had op zijn beurt een opregulatie van *Dcr2*, *Ago2* en *BmdsRNase* tot gevolg. Soaking van de BmToll9-1 getransformeerde cellen in dsRNA toonde een verhoogde transcriptionele respons aan van de meeste immuungenen betrokken in de verschillende immuun transductie pathways en *Dcr2* in de RNAi pathway. Deze resultaten dragen bij tot de kennis van de RNAi machinerie pathways en de immuun signalisatie pathways en leveren elementen aan waarmee rekening moet gehouden worden bij het opzetten van RNAi experimenten in lepidopteren zoals *B. mori*.

Curriculum vitae

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A1 Peer reviewed publications

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