Of Insects and Viruses: The Role of Small RNAs in Insect Defence

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Abstract

In the past decade, small RNA pathways have been identified as a major mechanism of gene regulation. From an immunity standpoint, these pathways play a central role either by regulating immune reactions or by acting as immune effectors. In insects, several studies have unravelled the role of RNA interference (RNAi) as an antiviral response and have uncovered a complex relationship between insects and viruses that co-evolve in an ongoing race for supremacy. In this review, we comment on the role of small RNA pathways in insect defence and the exploitation of these same pathways by pathogens. We illustrate the host–pathogen relationship under RNAi constraints using several examples and we discuss future directions in using RNAi as a tool to control insect immunity.

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1 Introduction

Host–pathogen interactions can be pictured as an arms race between two adversaries. On the one hand, the pathogen deploys virulence factors to exploit the resources of the host. On the other hand, the host fights back with immune responses to clear the pathogen or at least minimise its deleterious effects. The outcome of this interaction can vary from commensalism to the death of one of the two players, depending on the relative strength of the effectors involved and possible escape mechanisms to limit these effects. There are two types of immune responses, relying on different mechanisms and effectors: innate and adaptive. While the latter is specific to vertebrates, innate immunity is present in all multicellular organisms, including insects. In vertebrates, the activation of the innate immune response is a prerequisite to the activation of adaptive immunity. Two theories have been proposed to model the induction of the innate immune response. The first theory relies on the recognition by Pattern Recognition Receptors (PRRs) of invariant molecular patterns (Pathogen-Associated Molecular Pattern—PAMP) that are present in most, if not all, microbes (Janeway, 1989, 1992). According to the second theory, the 'theory of danger', the immune system is elicited by alarm signals sent by injured cells (Matzinger, 1994). These two theories are not mutually exclusive as illustrated by the dual activation of *Drosophila* immune response to both glucans (PAMP) and virulence factors (alarm signal) upon infection by the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Gottar *et al*., 2006). Recently, Polly Matzinger reformulated her initial theory of danger, unifying the recognition by PRRs and the recognition of alarm signals into a single model in which the immune system responds to 'danger signals' from different origins and sources (Matzinger, 2007).

1.1 INSECTS AND VIRUSES

Insects, like other organisms, are subject to infection by viruses with RNA or DNA genomes of different structure and polarity. These viruses fall into two distinct classes, depending on the type of host they use during their replication cycle. Viruses from the first class are transmitted from insect to insect, although their host range may not be solely restricted to insects. Among these viruses, some present a strong agronomical impact by affecting domesticated insects. For instance, the Israeli Acute Paralysis Virus (IAPV) is among the pathogens potentially responsible for the honeybee (*Apis mellifera*) colony collapse disorder, which results in the destruction of the hives (Cox-Foster *et al*., 2007). Others, like *Densovirus* (Parvoviridae) have been successfully used to eliminate *Galleria mellonella* (greater wax moth) infestations in beehives (Lavie *et al*., 1965), although the use of viruses for biological control of pests remains limited because of their potential impact on human health.

Viruses of the second class are the arthropod-borne viruses (arboviruses). These viruses have the particularity of alternating between hematophagic invertebrate and vertebrate hosts in an obligate fashion. Several insects are responsible for arbovirus transmission to human or cattle, including the mosquitoes *Aedes* spp. (e.g. Rift Valley Fever Virus, Chikungunya Virus, Dengue Virus, Yellow Fever Virus), *Anopheles* spp. (e.g. O'nyong'nyong Virus) and *Culex* spp. (e.g. Rift Valley Fever Virus, Japanese Encephalitis Virus, West Nile Virus); the sand fly *Phlebotomus* spp. (e.g. Vesicular Stomatitis Virus, VSV) and biting midges in the genus *Culicoides* (e.g. Bluetongue Virus; for review see Mellor, 2000 and references therein). Importantly, arboviral infections are asymptomatic in insects but responsible for severe incapacitating diseases in mammalian hosts, especially humans, suggesting a complex co-evolutionary process. There is no specific treatment against such diseases and vaccines are available for only two of them (the Yellow Fever and Japanese Encephalitis viruses). The strong impact of arboviruses on human health and economy, the spread of these diseases around the globe due to climate changes and travelling habits, and the emergence of new arboviral diseases make clear the importance of finding new strategies to limit arboviral transmission to mammals. More generally, insects play important roles in human life, both beneficial and detrimental; this prompts a desire for better understanding of their immune system in order to protect those that are valuable to humans and limit those that bring adverse effects.

1.2 *DROSOPHILA* AS A MODEL TO STUDY HOST–PATHOGEN INTERACTIONS

Upon viral infection, insects mount a distinctive immune response whose hallmark is RNA interference (RNAi). This defence mechanism was originally identified as an antiviral defence in plants (Ratcliff *et al.*, 1997). In insects, most of our knowledge about RNAi and more generally about small RNA pathways come from studies performed in the fruit fly *Drosophila melanogaster*, which celebrated its hundredth anniversary as a model organism in 2010. Since its introduction in the laboratory by Thomas Morgan to study development, *Drosophila* has been the source of invaluable contributions to genetics, developmental biology, neuroscience and immunology. For instance, the first mutation described by Morgan in 1910 led to the discovery of sex-linkage, introduced genetics as a science and genetic analysis as a powerful tool in biology. Throughout the century, *Drosophila* has benefited from a dynamic and collaborative community that rendered this small fly one of the most accomplished multicellular animal models in which to carry out genetic analysis. In addition, the sequence of its genome showed that more than 60% of the genes involved in human genetic diseases are conserved in *Drosophila* (Bier, 2005). Thus, its affordability, short generation time and amenability to both direct and reverse genetics makes it suitable for intensive analysis and provides an appropriate alternative to vertebrate models when characterizing biological processes.

The major breakthrough in immunology that can be attributed to *Drosophila* is the identification of the Toll (Lemaitre, 2004; Lemaitre *et al*., 1995b, 1996) and Imd (Lemaitre *et al*., 1995a) pathways which direct an immune response capable of discriminating and taking the appropriate action against an invading microbe, depending on its class (Lemaitre *et al*., 1997). The identification of Toll pioneered the discovery of the Toll-like receptors (TLR) in mammals and the subsequent understanding of the mechanisms that govern innate immunity (Rock *et al*., 1998). More recently, the identification of the RNAi pathway as the major antiviral defence mechanism positioned *Drosophila* as a central model to study insect antiviral immunity (Galiana-Arnoux *et al*., 2006; van Rij *et al*., 2006; Wang *et al*., 2006; Zambon *et al*., 2006). In the case of arboviruses, enthusiasm for *Drosophila* as a study model over genuine vectors such as mosquitoes may be dampened by the fact that it is not a vector for any arboviral disease. However, the ability of several arboviruses to replicate in *Drosophila* (e.g. Sindbis Virus (SINV): Xiong *et al*., 1989; West Nile Virus: Chotkowski *et al*., 2008; Dengue Virus: Sessions *et al*., 2009; VSV: Mueller *et al*., 2010) and the quasi-absence of genetic tools available in other insects make *Drosophila* a very powerful model to study all virus–insect, and more specifically arbovirus–insect, interactions.

2 Generalities about insect defence mechanisms

Insects have developed effective defence mechanisms to protect themselves from infections. These defence mechanisms rely on both physical and chemical barriers that prevent microbes from penetrating the body cavity, along with a wide range of inducible reactions that aim at eradicating invading microorganisms. Below, the main aspects of the antimicrobial and antiviral responses in *Drosophila* are summarised; these are likely to be conserved in other insects.

2.1 THE ANTIMICROBIAL IMMUNE RESPONSE

First of all, the cuticle, which covers the insect body, prevents the entry of microbes into the body cavity through the epidermis. The epithelia of the intestinal and respiratory tracts (trachea) are also lined by chitinous membranes that avert direct contact between cells and microbes. In the gut, which constitutes the main route of infection, the secretion of digestive enzymes, a low pH and the production of reactive oxygen species maintain an environment hostile to microbial survival (Lemaitre and Hoffmann, 2007; Tzou *et al*., 2002). When microbes overcome this first line of defence, the tracheal and intestinal cells secrete antimicrobial peptides (AMPs) to eradicate microbes that would persist and colonise the lumen of their respective epithelia (Tzou *et al*., 2000).

Once these physical and chemical barriers are breached, the entry of microbes within the body cavity triggers immune reactions with cellular and humoral components. The cellular response mainly involves macrophages, called plasmatocytes,

that engulf microbes present in the circulatory system or hemolymph through phagocytosis (for review see Lemaitre and Hoffmann, 2007). Microbes also induce a humoral response whose hallmark is the synthesis and secretion of several AMPs by the fat body. The expression of AMPs is regulated by two parallel NF-κB signalling cascades, the Toll and Imd pathways, whose intracellular components display striking similarity with those of the vertebrate TNF-R (Tumour Necrosis Factor Receptor) and TLR/IL-12R (Interleukin 12 Receptor) pathways, respectively (Lemaitre and Hoffmann, 2007). The Imd pathway is activated predominantly by Gram-negative bacteria and in turn, activates the expression of antibacterial peptide-encoding genes (e.g. *diptericin*), whereas the Toll pathway is predominantly activated by Gram-positive bacteria and fungi, and regulates the expression of genes that encode antifungal peptides (e.g. *drosomycin*) and a subset of antibacterial peptides (Lemaitre and Hoffmann, 2007; Lemaitre *et al*., 1997). These two pathways are discriminatively activated through the recognition of a small repertoire of PAMPs which consist of a peptidoglycan whose structure differs between Gram-positive and Gram-negative bacteria (Leulier *et al*., 2003), and the fungal beta-1,3 glucans (Gottar *et al*., 2006). Although the two pathways are down-regulated to limit the adverse effects of prolonged activation (Zaidman-Remy *et al*., 2006), AMP concentrations are sustained at high levels over several days, protecting the flies against a second challenge (Boman *et al*., 1972).

2.2 THE ANTIVIRAL RESPONSE

The core antiviral response of *Drosophila* is distinct from its antimicrobial counterpart. It involves different components that can be divided into two classes depending on the mechanisms by which they are elicited. The first class is directly triggered by the presence of double-stranded RNA (dsRNA) and includes the degradation of the viral genome by the RNAi machinery (Galiana-Arnoux *et al*., 2006; van Rij *et al*., 2006; Wang *et al*., 2006; Zambon *et al*., 2006) and the regulation of genes like *Vago* via an RNAi-independent function of Dicer-2 (Dcr-2; Deddouche *et al*., 2008). The second class is more likely to respond to cell damages and involves the Jak/STAT pathway (Dostert *et al*., 2005) and potentially, the Toll and Imd pathways (Avadhanula *et al*., 2009; Costa *et al*., 2009). The RNAi pathways will be extensively discussed in the Section 3; below the role of other pathways in the insect antiviral response is summarised.

The Jak/STAT signalling cascade is involved in numerous developmental processes (for review see Arbouzova and Zeidler, 2006). This pathway is constituted in *Drosophila* by cytokines of the Unpaired family, the transmembrane receptor domeless (dome), the Janus Kinase hopscotch (hop) and the transcriptional activator STAT92. The Jak/STAT pathway was originally thought to be involved in the antimicrobial response (Agaisse *et al*., 2003); although mutations impairing the pathway do not affect AMP expression or fly survival after microbial infection (Dostert *et al*., 2005), while *hop*-deficient flies

display a strong susceptibility to *Drosophila* C virus (DCV) challenge. A similar antiviral role was proposed for the Jak/STAT pathway in the mosquito *Aedes aegypti* upon dengue infection (Souza-Neto *et al.*, 2009). In *Drosophila*, two lines of evidence suggest that the activation of the Jak/STAT pathway constitutes a secondary response to viral infection, possibly in response to cellular damage: (i) the expression of one of its virus-induced targets, *vir-1*, is upregulated in tissues in which virus is undetectable (Dostert *et al.*, 2005) and (ii) vir-1 is only expressed when flies are infected with live virus but not after injection of UV-inactivated virus or viral dsRNA (Hedges and Johnson, 2008). These latter results correlate with the inability of a constitutively active allele of *hop*, *Tum-l*, to activate *vir-1* expression in the absence of viral infection (Dostert *et al.*, 2005). Taken together, these data suggest that the Jak/STAT pathway responds to cytokine signalling thereby informing cells of viral infection. This role is reminiscent of that of the Jak/STAT pathway in mammals, illustrating the conservation of innate immunity regulatory mechanisms through evolution.

Recently, the antimicrobial pathways Toll and Imd were reported to play an antiviral role in *Drosophila* (Avadhanula *et al.*, 2009; Costa *et al.*, 2009) and the mosquito *A. aegypti* (Xi *et al.*, 2008). However, the reported results appear contradictory possibly because they were obtained from different host-virus interaction models. Because the role of the Toll and Imd pathways in antiviral response is beyond the scope of this review, we will not further discuss these results.

3 RNAi and the immune response

RNAi refers to sequence-specific RNA-dependent silencing mechanisms (Fire *et al.*, 1998; Ratcliff *et al.*, 1997) that regulate various processes such as gene expression, epigenetic modifications and defence against pathogens. These mechanisms are conserved throughout evolution and functional RNAi pathways have been identified in fungi, plants and animals albeit with different roles. Almost all RNAi pathways are triggered by dsRNA that varies in length and origin. The dsRNA molecules are rapidly converted by RNaseIII enzymes called Dicers into small RNAs whose sizes range from 21 to 32 nucleotides, depending on the pathway from which they originate. Next, the small RNAs are loaded into a multiprotein RNA-Induced Silencing Complex (RISC) where they guide the recognition of target RNA through an Argonaute (AGO)/Piwi family member. These AGO/Piwi proteins constitute the heart of the RNAi system, as they bind small RNAs and directly mediate silencing at the transcriptional and post-transcriptional levels.

3.1 THE SMALL RNA PATHWAYS

There are three classes of small RNAs that differ in size, the template from which they originate and the pathway through which they are processed (Fig. 1).

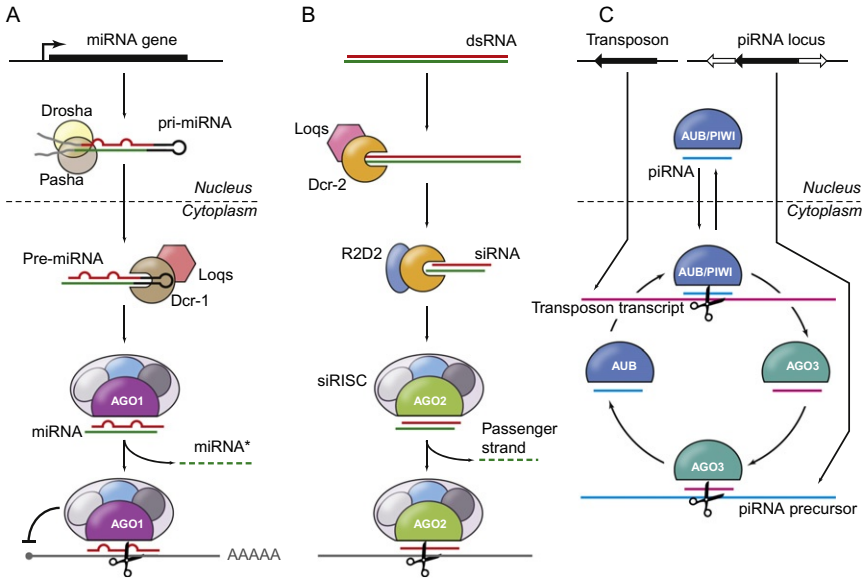

FIG. 1 Schematic representation of the small RNA pathways in *Drosophila*. (A) The miRNA pathway is initiated by the transcription of miRNA genes. Primary miRNA (pri-miRNA) transcripts are first processed by the Drosha/Pasha complex in the nucleus then exported to the cytoplasm as pre-miRNAs. There, Dcr-1 and loquacious (loqs) complete the processing and deliver the mature miRNA to AGO1-containing RISC. The miRNA* (star) is eliminated and the guide miRNA directs translational repression or cleavage of the cognate mRNA. (B) The siRNA pathway is initiated by double-stranded RNA of viral or genomic origin which is recognised and cleaved by Dcr-2 with the help of loqs. The resulting double-stranded siRNAs are delivered to AGO2-containing RISC by Dcr-2 and R2D2. The passenger strand is eliminated and the guide siRNA directs the degradation of the target RNA via AGO2 catalytic activity. (C) The piRNA pathway is initiated by maternally deposited piRNAs loaded into Aubergine (Aub)/Piwi proteins. Through an amplification loop that involves sense and antisense transcripts, AGO3 and Aub, the piRNA pool is amplified. The newly produced piRNAs loaded into Aub are transported to the nucleus where they are thought to be involved in chromatin modifications.

microRNAs (miRNAs; Fig. 1A) are 22 nucleotide-long small RNAs originating from longer genome-encoded precursors that contain an imperfectly base-paired hairpin. This hairpin is first processed by the Drosha/Pasha complex in the nucleus before being exported to the cytoplasm (Denli *et al*., 2004; Han *et al*., 2004; Lee *et al*., 2003). Then, Dicer-1 (Dcr-1) completes processing into miRNAs before loading them into AGO1-containing RISC where they guide translational repression and mRNA degradation through different mechanisms (for review Eulalio *et al*., 2008). miRNAs constitute the most evolutionarily conserved class of small RNAs and play a critical role in development and the regulation of cellular processes.

Small interfering RNAs (siRNAs; Fig. 1B) are 21 nucleotide-long small RNAs that originate from perfectly base-paired long dsRNA molecules that are processed through a Dcr-2/AGO2 pathway. The siRNA pathway is mostly involved in defence against parasitic nucleic acid elements, such as retrotransposons or viruses. Two subclasses of siRNA can be distinguished based on the origin of the dsRNA. endo-siRNAs are produced from genome-encoded inverted-repeated structures or sites of convergent transcription and antisense transcripts from various loci, including retrotransposons and pseudogenes. In *Drosophila*, endo-siRNAs are produced both in the soma and the gonads and are thought to be involved in genome stability by repressing transposition (Brennecke *et al.*, 2007; Chung *et al.*, 2008; Ghildiyal, *et al.*, 2008) and acting on heterochromatin maintenance (Fagegaltier *et al.*, 2009). Although the canonical Dcr-2/AGO2 pathway has not been yet identified in vertebrates, endo-siRNAs were detected in mouse oocytes where they are thought to participate in the control of retrotransposons and pseudogenes (Tam *et al.*, 2008; Watanabe *et al.*, 2008). vsiRNAs are virus-derived siRNAs that originate from viral genomes (dsRNA viruses), viral intermediates of replication (RNA viruses with the exception of retroviruses), or convergent overlapping transcriptional units (DNA viruses). vsiRNAs play a critical role in the antiviral response in insects, nematodes and plants, but to date, this role has not been shown to be conserved in vertebrates (Umbach and Cullen, 2009).

Piwi-interacting RNAs (piRNAs; Fig. 1C) are longer, small RNAs (24–32 nucleotides) and differ from the miRNAs and siRNAs in three main features. First, piRNAs are believed to be processed from single-stranded primary transcripts that are transcribed from defined genomic regions rather than dsRNA molecules (Aravin *et al.*, 2007a). Second, although their biogenesis is not completely understood, it only requires Piwi proteins and seems to be independent of Dicer (Houwing *et al.*, 2007; Vagin *et al.*, 2006). Third, they contribute to the silencing of transposable elements exclusively in the animal gonads (Aravin *et al.*, 2003, 2007b; Brennecke *et al.*, 2007; Olivieri *et al.*, 2010; Vagin *et al.*, 2006).

3.2 ANTIVIRAL RNAI IN INSECTS

RNAi was first identified as an antiviral defence mechanism in plants (Ratcliff *et al.*, 1997) and in the nematode *Caenorhabditis elegans* (Lu *et al.*, 2005; Schott *et al.*, 2005; Wilkins *et al.*, 2005). In insects, RNA-dependent silencing of viral replication was first described using recombinant SINV-expressing Dengue Virus 2 (DENV2) sequences from a duplicated subgenomic promoter. Mosquitoes infected with the recombinant SINV were resistant to a second challenge by the wild-type DENV2 through an RNA-dependent mechanism (Adelman *et al.*, 2001; Gaines *et al.*, 1996; Olson *et al.*, 1996). Similar results indicating an RNA-based mechanism were observed with recombinant SINV-expressing Rift Valley virus sequences (Billecocq *et al.*, 2000). The link

between the above observations and RNAi was established in *Drosophila* where the depletion of *AGO2* led to the accumulation of Flock House virus (FHV) RNAs in cell culture (Li *et al*., 2002); these observations were later confirmed in a mosquito (Keene *et al*., 2004). More recently, several studies confirmed the role of RNAi as an antiviral response in insects and the siRNA pathway as a major player in fighting against viral infection. Indeed, flies carrying null alleles of *Dcr-2*, *AGO2* or *R2D2* (see below for details) are more susceptible to viral infection as illustrated by their inability to control viral replication and by their premature death compared to wild-type individuals. Although RNAi has only been shown to exert an antiviral function in *Drosophila* (Galiana-Arnoux *et al*., 2006; van Rij *et al*., 2006; Wang *et al*., 2006; Zambon *et al*., 2006) and mosquitoes (Campbell *et al*., 2008; Cirimotich *et al*., 2009; Keene *et al*., 2004; Khoo *et al*., 2010; Sanchez-Vargas *et al*., 2009), several insects display a functional RNAi machinery presumably also involved in antiviral defence (Table 1).

From an evolutionary standpoint, some genes in the antiviral RNAi pathways are shown to display a high evolutionary rate compared to non-immune genes (Obbard *et al*., 2006). A similar observation was made about the genes that encode the intracellular components of the antimicrobial pathways (for review Lazzaro, 2008). These data have been interpreted as the result of a constant co-evolution between hosts and pathogens, the latter exerting suppressive strategies on host genes, forcing their adaptation (Lazzaro, 2008). Interestingly, among the strategies developed by viruses to escape from RNAi are the viral suppressors of RNAi (VSRs; for further details see Section 4.1). These VSRs affect various steps in the RNAi pathways and may explain the fast evolution of RNAi genes whose products are targeted by VSRs.

3.3 THE SIRNA PATHWAY: THE MAJOR ANTIVIRAL REACTION IN INSECTS

Among small RNA pathways, the siRNA pathway plays a major role in defence against viral infection. This pathway is composed of two core components that display antiviral function: Dcr-2 and AGO2-containing RISC.

3.3.1 *Dicer-2*

Upon viral infection, dsRNA is produced either as the viral genome itself or as an intermediate of replication. The viral dsRNA is first recognised and processed as double-stranded siRNA by Dcr-2 with the help of loquacious (Marques *et al*., 2010). From a structural point of view, Dcr-2 includes the following domains from its N- to its C-terminus: (i) a DExH-box helicase domain, (ii) two RNAseIII domains responsible for dicing of the dsRNA and (iii) a dsRNA-binding domain (Lee *et al*., 2004). Dcr-2 plays a fundamental role in antiviral defence through several aspects of its function. First, the cleavage of viral dsRNA by the RNase III activity is in itself an antiviral mechanism (Flynt

TABLE 1
Examples of insects displaying an RNAi response *in vivo* following uptake of exogenous dsRNA[a]

	Silencing occurs in:
Blattaria	
Blattella germanica	Epidermis, thoracic gland and fat body (Martin *et al*., 2006)[b]
Diploptera punctata	Corpora allata (endocrine gland) (Lungchukiet *et al*., 2008)
Coleoptera	
Harmonia axyridis	Wing imaginal discs (Ohde *et al*., 2009)
Monochamus alternatus	Epidermis (Niu *et al*., 2008)
Sitophilus spp.	Bacteriome tissue (Vallier *et al*., 2009)
Tenebrio molitor	Hemolymph, presumably hemocytes or fat body (Zhao *et al*., 2005)
Tribolium castaneum	Progeny (Bucher *et al*., 2002)
Diptera	
Aedes spp.	Fat body (Bartholomay *et al*., 2004)
Anopheles gambiae	Fat body (Blandin *et al*., 2002)
Armigeres subalbatus	Hemocytes (Infanger *et al*., 2004)
Bactrocera dorsalis	Ovary (Chen *et al*., 2008)
Ceratitis capitata	Systemic (Vannini *et al*., 2010)
Culex pipens	Systemic (Sim and Denlinger, 2008)
Drosophila melanogaster	Central nervous system (Dzitoyeva *et al*., 2001)
Glossina spp.	Fat body (Lehane *et al*., 2008)
Lutzomyia longipalpis	Systemic (Sant'Anna *et al*., 2008)
Hemiptera	
Acyrthosiphon pisum	Ubiquitous and gut (Jaubert-Possamai *et al*., 2007)
Bemisia tabaci	Midgut and salivary glands (Ghanim *et al*., 2007)
Nilaparvata lugens	Ubiquitous, gut and central nervous system (Liu *et al*., 2010)
Planococcus citri	Embryo (soaking of embryos, Volpi *et al*., 2007)

Rhodnius prolixus	Salivary glands (injection and ingestion, Araujo *et al*., 2006)
Triatoma brasiliensis	Gut (Araujo *et al*., 2007)
Hymenoptera	
Apis mellifera	Fat body (Amdam *et al*., 2003)
Nasonia vitripennis	Progeny (Lynch *et al*., 2006)
Lepidoptera	
Bombyx mori	Silk gland (Tabunoki *et al*., 2004)
Epiphyas postvittana	Gut (ingestion, Turner *et al*., 2006)
Helicoverpa armigera	Midgut (Sivakumar *et al*., 2007)
Hyalophora cecropia	Ovary/embryo (Bettencourt *et al*., 2002)
Manduca sexta	Fat body and hemoytes (Eleftherianos *et al*., 2006)
Spodoptera spp.	Midgut (Rajagopal *et al*., 2002)
Orthoptera	
Gryllus bimaculatus	Systemic (Meyering-Vos *et al*., 2006)
Locusta migratoria	Progeny (He *et al*., 2006)
Schistocerca spp.	Eye (Dong and Friedrich, 2005)

[a] This list does not consider experiments involving injection of dsRNA in pre-blastoderm embryos as they do not reflect dsRNA uptake.
[b] Given the extensive literature available on this subject, we have only considered the first publication, to our knowledge, that reported an RNAi response *in vivo*. In all the experiments listed dsRNA was administered by intra-hemocoelic injection at larval or adult stages unless otherwise stated.

et al., 2009). Indeed, the degradation of dsRNA reduces the amount of template available for the production of new genomes or RNA that encode viral proteins. Second, the production of siRNA is absolutely required for subsequent steps in the RNAi pathway including the formation of an active RISC complex (see below). Third, Dcr-2 is involved in the regulation of some antiviral genes including *Vago* (Deddouche *et al*., 2008). This role of Dcr-2 in regulating, directly or indirectly, an antiviral transcriptional response is proposed to be dependent on the helicase domain of Dcr-2. However, none of the *Dcr-2* alleles used in this study produced siRNA (Lee *et al*., 2004) and it thus remains to be established whether the virus-dependent induction of *Vago* is regulated by the sole recognition of dsRNA by Dcr-2, its dicing activity or the presence of viral siRNA. Interestingly, as observed with *vir-1*, *Vago* is not upregulated by the sole presence and processing of dsRNA but requires viral replication, suggesting a more complex mechanism of regulation. Together, these data show that Dcr-2 is a PRR, capable of sensing and processing dsRNA as a signature of viral infection and of initiating both an RNAi-dependent and an RNAi-independent response.

3.3.2 *RNA-induced silencing complex*

Once cleaved from a dsRNA precursor by Dcr-2, double-stranded siRNAs are transferred by Dcr-2 to siRISC in an asymmetric manner (Schwarz *et al*., 2003; Tomari *et al*., 2004) with the help of R2D2 (Liu *et al*., 2003, 2006). RISC is a ribonucleoproteic complex that contains several components including AGO2 (Hammond *et al*., 2001), dFXR (Drosophila ortholog of fragile X mental retardation protein, Caudy *et al*., 2002), VIG (vasa intronic gene, Caudy *et al*., 2002) and siRNA, while other components promote siRISC assembly/activation including aubergine (Specchia *et al*., 2008), C3PO (Liu *et al*., 2009) or Hsp90 (Miyoshi *et al*., 2010). Once the double-stranded siRNA is loaded into siRISC, one of the two strands, called the passenger strand, is eliminated through its cleavage by AGO2 (Matranga *et al*., 2005; Rand *et al*., 2005). The antiviral activity of the resulting active siRISC resides in its ability to 'search and destroy' target/viral RNAs and to degrade them in a sequence-specific fashion (Liu *et al*., 2004). The specificity of this mechanism is provided by the perfect base-pairing between the remaining siRNA guide strand and its target which precedes the cleavage of the target RNA by AGO2 at position 10 with respect to the 5′ extremity of the siRNA. Importantly, the guide strand-assisted cleavage activity of AGO2 is strongly dependent on the perfect base-pairing between the siRNA and its target. Indeed, mutations located around position 10 strongly affect AGO2 cleavage activity and it is believed to strongly impair its antiviral activity (Boden *et al*., 2003; Das *et al*., 2004; Gitlin *et al*., 2005; Wilson and Richardson, 2005 and see Section 4.2).

Recently, it was also shown that AGO2 can also repress translation (Iwasaki *et al*., 2009). Although AGO2 inhibition of translation has not been studied in

the context of the antiviral response, it is tempting to think that such a mechanism could also account for AGO2 antiviral activity in insects.

3.3.3 *Other players*

Recently, a link between the siRNA and the miRNA pathways in antiviral response was proposed with the identification of Ars2 and the nuclear proteins CBP20 and CBP80 as components of the small RNA pathways (Sabin *et al*., 2009). Flies deficient for either gene display an increased susceptibility to viral infection with DCV or VSV. While these genes interact with components of the miRNA (microprocessor) and siRNA (Dcr-2) pathways, the relevance of each pathway in the phenotypes observed and the role of these genes in controlling viral replication independently of the small RNA pathways remain to be established.

3.3.4 *Amplification: Making silence louder*

The mechanism of RNAi amplification is a specific feature of organisms whose genomes encode RNA-dependent RNA polymerases (RDRs[1]) such as plants (Vaistij *et al*., 2002; Voinnet *et al*., 1998) and *C. elegans* (Sijen *et al*., 2001). Amplification was first discovered through a process called transitivity. In transitivity, aberrant ssRNA transgene-derived transcripts are converted by RDR into dsRNA molecules that are processed into siRNA by Dicer proteins. The resulting siRNAs guide the synthesis of dsRNA from an ssRNA template (Alder *et al*., 2003; Makeyev and Bamford, 2002; Pak and Fire, 2007). These secondary dsRNAs then produce secondary siRNAs, which in the case of transgene-induced silencing, extend over the originally targeted sequence (Fig. 2). So far, the importance of amplification in antiviral defence has only been demonstrated in plants as illustrated by the increased susceptibility of RDR mutants to viral infection (Schwarz *et al*., 2003; Wang *et al*., 2010; Xie *et al*., 2001). Of note, as RDR are mandatory for RNAi in *C. elegans* (Sijen *et al*., 2001; Smardon *et al*., 2000), the role of amplification cannot be uncoupled from RNAi itself.

In *Drosophila*, the existence of transitivity was addressed by testing the production of secondary siRNAs directed against Green Fluorescent Protein (GFP) fusion constructs while targeting the GFP sequence by primary siRNAs produced from a genome-encoded hairpin (Roignant *et al*., 2003). Under these conditions, the authors did not identify secondary siRNA, suggesting the absence of transitivity and thus amplification in *Drosophila*, consistent with the absence of a canonical RDR-encoding gene in this organism (Roignant *et al*., 2003) and in insects in general. However, the recent identification of an RDR activity fostered by the largest subunit of the *Drosophila* RNA polymerase

[1] Of note, eukaryotic RNA-dependent RNA polymerase is abbreviated as RDR while those of RNA viruses are abbreviated as RdRp.

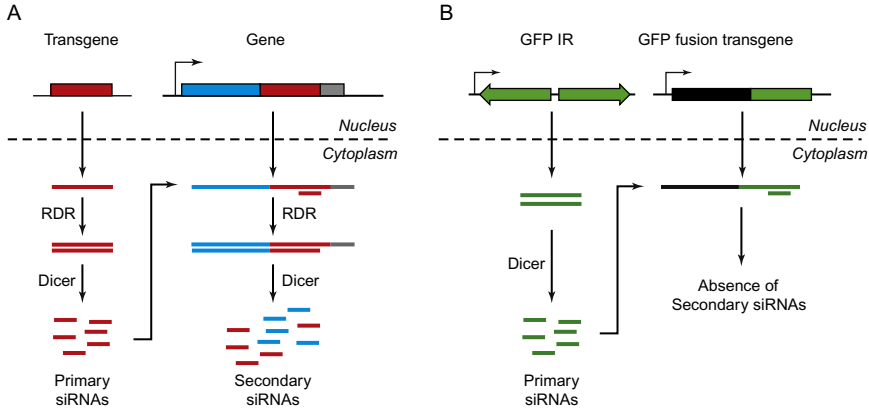

FIG. 2 Examples of transitivity assays in plants (A) and *Drosophila* (B). (A) In plants, aberrant transcripts produced from an integrated transgene corresponding to a fragment of an endogenous gene are converted into dsRNA by an RNA-dependent RNA polymerase (RDR) and cleaved by Dicer. The resulting siRNAs (red) are used to prime the RDR-dependent synthesis of a dsRNA molecule using the homologous endogenous gene transcript as template. Consequently, the newly synthesised dsRNA molecule contains sequence of the surrounding transcript (blue). The presence of secondary siRNAs corresponding to the surrounding regions (blue) is a signature of transitivity. (B) A similar assay was designed in *Drosophila* but failed to identify secondary siRNAs. An inverted repeat (IR) directed against the GFP (green) was expressed from the fly genome. GFP siRNAs were detected (green). However, secondary siRNAs corresponding to the surrounding sequences (black) of a GFP fusion transcript were not detected arguing against transitivity in *Drosophila*.

II elongation factor *in vitro* (D-elp1, Lipardi and Paterson, 2009) reopened the debate on transitivity and amplification in insects. Indeed, several lines of evidence suggest a role of D-elp1 in RNAi: (i) depletion of *D-elp1* in *Drosophila* S2 cells reduces siRNA-mediated gene silencing when cells are soaked with dsRNA, (ii) this depletion is accompanied with an over-representation of transposon-derived transcripts and (iii) immunoprecipitation experiments suggest that D-elp1 interacts with Dcr-2 consistent with a cytoplamic localisation of D-elp1 (Lipardi and Paterson, 2009). Nevertheless, several questions must be addressed, among others: (i) does D-elp1 exhibit an RDR activity *in vivo*?, (ii) is this RDR activity required for D-elp1 function in RNAi? and (iii) in which RNAi-based process (e.g. genome stability through transposition silencing, antiviral defence) is D-elp1 required?

3.3.5 *Systemic RNAi: Sharing the silence*

In plant and worms, the effects of RNAi are not only cell autonomous but spread across the entire organism away from the initial site of silencing (for review Voinnet, 2005). However, the transport of the silencing signal differs drastically

in these two systems. In plants, the silencing signal (small RNAs) is spread via plasmodesmal junctions that link almost all the cells together (short-distance spread) and the vascular system (phloem, long-distance spread) while in *C. elegans*, the cell-to-cell spread of the RNAi effect relies on transmembrane transporters of the SID family (Feinberg and Hunter, 2003; Winston *et al*., 2002, 2007). The spread of RNAi plays a critical role in antiviral defence in plants by initiating an RNAi response against the virus in the entire organism. This protects uninfected cells from infection and thus confines the deleterious effects of viral infection to a limited number of cells. In *Drosophila*, the absence of *sid-1*-related genes and the strict cell autonomy of RNAi effects when initiated from genome-encoded hairpin constructs (Roignant *et al*., 2003) led to the belief that insects lacked a systemic RNAi pathway. However, the administration of exogenous dsRNA by injection was reported to induce gene knockdown in a growing number of insects from various orders including Diptera, Hymenoptera, Hemiptera, Coleoptera, Lepidoptera and Orthoptera (Table 1). In addition, similarly to *C. elegans* (Timmons and Fire, 1998), some insects are capable of initiating an RNAi response after ingestion of dsRNA, including the honeybee (Patel *et al*., 2007), the beet armyworm *Spodoptera exigua* (Tian *et al*., 2009), the brown planthopper *Nilaparvata lugensthe* (Chen *et al*., 2010), the light brown apple moth *Epiphyas postvittana* (Turner *et al*., 2006), the tobacco hornworm *Manduca Sexta*, the red flour beetle *Tribolium castaneum* and the pea aphid *Acyrthosiphon pisum* (Whyard *et al*., 2009). Finally the genomes of several insects including the honey bee, the parasitic wasp *Nasonia vitripennis*, the silkworm *Bombyx mori*, the aphid *Aphis gossypii* and *T. castaneum* encode *sid-1*-like genes, while these genes are absent from other insects including *Drosophila* and mosquitoes (Tomoyasu *et al*., 2008; Xu and Han, 2008). However, it remains unclear whether these genes play a role in systemic RNAi. Together, these findings show that insect cells are able to take up dsRNA and also suggest that systemic RNAi may occur experimentally in some insects.

More recently, two studies reported further evidence strongly suggesting systemic antiviral RNAi spread upon viral infection *in vivo* in *Drosophila* (Saleh *et al*., 2009) and *in vitro* in *Aedes albopictus* cells (Attarzadeh-Yazdi *et al*., 2009). Attarzadeh-Yazdi and colleagues showed spread of an 'RNAi signal' most likely transported from cell-to-cell in mosquito cell culture after infection with Semliki Forest virus (Attarzadeh-Yazdi *et al*., 2009). In adult *Drosophila*, it was proposed that upon viral infection, cells release an 'RNAi signal' through either cell lysis or membrane shedding that is taken up by surrounding uninfected cells to initiate an immune RNAi antiviral response. Impairing the dsRNA uptake pathway in flies dramatically increased susceptibility to viral infection and inability to control viral replication (Saleh *et al*., 2009). While the spreading signal remains to be characterised, previous studies *in vitro* show that *Drosophila* hemocyte-derived S2 cells have an active and selective mechanism for uptake of only long dsRNA from the surroundings

(Saleh *et al*., 2006). Based on the results provided by the studies in *Drosophila*, it appears that the spread signal is likely conveyed through the hemolymph rather than by cell-to-cell transport, although this hypothesis remains to be tested. Further experiments are also required to confirm the type of RNA molecules that are spread *in vivo*, to determine the range of action of systemic RNAi and to identify possible proteins involved in RNA transport.

3.4 OTHER DEFENCE MECHANISMS INVOLVING SMALL RNAS

3.4.1 *Other potential role for siRNAs*

In addition to its direct antiviral activity, the siRNA pathway may be involved in self-regulatory feedback loops aimed at boosting the antiviral response. For instance, in *Drosophila* the *AGO2* and *CG7739* genes overlap at the level of their respective 3′UTRs (Flybase at http://www.flybase.org). Interestingly, small RNA profiling reveals the presence of siRNA produced from a putative dsRNA that would correspond to the base pairing of the 3′UTRs of these two genes (B. Berry and C. Antoniewski, personal communication). A possible interpretation is that upon viral infection, RNAi would be reoriented towards the degradation of the viral genome titrating out endo-siRNAs and therefore reducing the impact of *AGO2*/*CG7739* repression by endo-siRNAs. As a result, the *AGO2* transcript would be less targeted for degradation and the AGO2 protein level would increase together with the efficiency of the RNAi response. This model is theoretical and requires further investigation.

3.4.2 *The role of miRNAs in insect immune response*

3.4.2.1. Cellular miRNAs and insect defence miRNAs have emerged as an important class of positive and negative regulators involved in multiple facets of both the innate and adaptive immune response in mammals. Indeed, while the miR-17–92 cluster, miR-150, miR-155, miR-181 and miR-223 are involved in the maturation, proliferation and differentiation of T-cells and B-cells, miR-9, miR-146a and miR-155 are believed to negatively regulate acute immune responses through the down-regulation of proteins involved in the receptor-induced signalling pathways (e.g. TLR, for review Tsitsiou and Lindsay, 2009). In insects, little is known about the regulation of immune pathways by miRNA. However, prediction of miRNA targets in *Drosophila* suggests that some immune-related genes, like *Imd*, could be regulated by miRNA (Microcosm at http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). Nevertheless, experimental validation of this hypothesis is required. In addition, several components of RNAi pathways are suggested as potential miRNA targets in vertebrates (John *et al*., 2004). In the case of the antiviral response, the possible involvement of miRNA in regulating the siRNA pathway would add another layer of small RNA regulation to this already complex network.

3.4.2.2. Viral-encoded miRNAs Viral-encoded miRNA were first identified in mammalian viruses including Adenovirus, Polyomavirus (e.g. SV40) and Herpesvirus (e.g. Epstein Bar virus, Kaposi's sarcoma-associated herpesvirus or human cytomegalovirus) where they regulate the host and their own gene expression to modulate the host response or control, for instance, the transition between latent and lytic cycles (for review Cullen, 2010). Recently, miRNAs derived from an insect baculovirus were identified and predicted to target both viral and host transcripts (Singh *et al*., 2010). However, the role of these miRNAs in the physiopathology of baculovirus infection remains to be investigated. Interestingly, viral-encoded miRNAs have only been identified in DNA viruses with a nuclear stage. Although viral miRNAs have been predicted in the genome of RNA viruses (Li *et al*., 2008), a study by Pfeffer and colleagues failed to identify any miRNAs produced by the Yellow Fever virus or the hepatitis C virus (Pfeffer *et al*., 2005). Another study identified small RNAs produced in mammalian cells after infection by various RNA viruses (Parameswaran *et al*., 2010). However, it remains to be established whether these virus-derived small RNAs are siRNAs, miRNAs or degradation products. One can argue that the absence of miRNAs in RNA viruses is due to replication occuring in the cytoplasm, away from Drosha and Pasha which are essential for the biogenesis of host-encoded miRNA. However, it was shown recently that the Epstein Bar virus-encoded miRNA BART2 can be fully processed in the cytoplasm of mammalian cells when expressed by the tick-borne encephalitis virus in a Drosha-independent fashion (Rouha *et al*., 2010). Thus RNA viruses may support miRNA production and processing but their existence remains to be demonstrated.

3.4.2.3. Viral hijacking of cellular miRNAs It was shown that viruses could exploit cellular miRNAs for their own benefit. For instance, contrary to the seasonal flu viruses, the H1N1 virus responsible for the Spanish flu in 1918 upregulates the expression of miRNAs in the lung that are involved in the regulation of immune reactions and cell death (Li *et al*., 2010). Along the same line, VSV upregulates the expression of several miRNAs upon infection of mammalian cells. In particular, the VSV-induced over-expression of miR706 inhibits VSV-induced cells death by limiting caspase-3 and -9 activation. Interestingly, as an arbovirus, VSV also infects insects. The potential role of VSV-induced miRNAs in down-regulating insect immunity would provide clues about the evolutionary strategies developed by these viruses as adaptations to disparate vertebrate and invertebrate host.

3.4.2.4. Other possible roles for the miRNA pathway in insect defence The miRNA pathway may directly contribute to the insect antiviral response. Two recent articles reported that siRNAs generated from imperfectly base-paired long dsRNA molecules are preferentially loaded into AGO1 rather than AGO2 (Ghildiyal *et al*., 2010; Tomari *et al*., 2007). In *in vitro* conditions, these mismatched siRNAs are thought to act as miRNAs.

In *Dcr-2* mutant flies infected with VSV, a peak of 21 nucleotide-long VSV-derived small RNAs has been observed (Mueller *et al*., 2010). This result suggests that another dicing activity produces siRNA-like small RNAs in the absence of Dcr-2. This could be achieved by the other known Dicer protein in *Drosophila*, Dcr-1, which has been thought to be dedicated to miRNA biogenesis. Unfortunately, the *Dcr-1* and *AGO1* mutants in *Drosophila* are embryonic lethal and thus non-compliant to any analysis. Nevertheless, it is possible that the absence of Dcr-2 may have revealed a role of Dcr-1 in antiviral response, although this role would be minor compared to that of the siRNA pathway.

3.4.3 *A potential role for piRNA in antiviral defence*

Little is known about the role of piRNA outside the context of controlling transposition in the gonads. Two early reports showed that *piwi*-family mutants (*piwi* and *aubergine*) in *Drosophila* (Zambon *et al*., 2006) were more sensitive to viral infection and that *Anopheles gambiae* in which *Ago3* had been knocked down displayed increased viral titres when compared to wild-type individuals (Keene *et al*., 2004). More recently, the production of long viral-derived small RNAs has been reported upon viral infection in *Drosophila* ovarian somatic sheet cells (OSS, Wu *et al*., 2010) and in the *A. albopictus* C6/36 cell line (Brackney *et al*., 2010; Scott *et al*., 2010). While further demonstration will be necessary to assess the nature of these small RNAs in both systems, they share striking features with endogenous piRNAs: (i) they are 25–30 nucleotide long with a size distribution peaking at 27 and 28, (ii) they are strand biased, with most of the reads matching against one strand of the viral genome; this is unlike viral-derived siRNAs and (iii) they display a strong nucleotide bias as previously described for endogenous piRNAs (Brennecke *et al*., 2007). However, while OSS cells only produced primary piRNA after infection (Wu *et al*., 2010), C6/36 cells produced primary and secondary piRNAs (N. Vodovar, unpublished observation based on analysis of the data published in Brackney *et al*., 2010). Although these observations evoke a possible gonadal origin for C6/36 cells, Scott and colleagues proposed a somatic origin for these cells with the lack of Dcr-2 activity compensated by activation of the piRNA pathway (Scott *et al*., 2010).

Together, these results strongly suggest that the piRNA pathway is triggered and protects against viral infection. Thus the piRNA pathway should be considered as part of the antiviral RNAi response. Of note, C6/36 cells have been reported to silence viral replication in an RNAi-mediated manner (e.g. Adelman *et al*., 2002), even in the absence of Dcr-2 activity (Scott *et al*., 2010). Therefore, we would like to stress that contrary to the conclusion of two recent studies (Brackney *et al*., 2010; Scott *et al*., 2010) this cell line displays a functional antiviral RNAi response.

3.4.4 *RNAi-based methods to prevent insect infection: were scientists caught off-guard?*

Insects and humans interact in a very complex fashion: some insects are domesticated for products they generate (e.g. honey or silk), some insects are disease vectors (e.g. mosquitoes) and some are agricultural pests (e.g. aphids). Ideally, one would hope to protect useful insects from infection, limit arboviral replication in vectors and control pests, all with fewer adverse effects than chemical pesticides. In the recent years, given the roles of RNAi in gene silencing and antiviral responses in insects, several strategies were proposed to achieve these goals. For instance, feeding pests with dsRNA targeting vital genes was demonstrated to be efficient in laboratory conditions (Whyard *et al*., 2009) as a proof of principle for the development of insect-specific insecticides.

As mentioned earlier, the IAPV is potentially responsible for colony collapse disorder (CCD, Cox-Foster *et al*., 2007). It was recently shown that feeding bees with dsRNA directed against the IAPV successfully reduced the effect of an IAPV infection (Maori *et al*., 2009). However, while this approach is appealing as a way to protect beehives against CCD, it cannot be extended to non-social insects. Along the same line, the immunisation of either *Drosophila* or mosquitoes with dsRNA corresponding to a fragment of a virus, protected the insects against a challenge with the corresponding virus (Keene *et al*., 2004; Saleh *et al*., 2009). However, as a general strategy, the individual immunisation of arboviral vectors in the wild by injecting protective dsRNA is obviously an infeasible task. A more practical approach was developed based on direct production of an antiviral dsRNA by the mosquito. This was achieved by generating transgenic *A. gambiae* carrying a fragment of the Dengue 2 virus genome expressed as an inverted repeat (Franz *et al*., 2006). While such transgenic mosquitoes are efficiently protected against Dengue 2 virus infection over 13 generations, this protective effect is reduced or eventually lost a few generations later through an uncharacterised mechanism (Franz *et al*., 2006, 2009). This approach is promising although obstacles remain, such as the release of transgenic animals in nature and the displacement of endogenous populations by virus-resistant strains.

Nonetheless, insects did not wait for scientist to develop such antiviral strategies. Indeed, several studies have highlighted the presence of cDNA sequences of non-retroviral RNA viruses in fungi, plant, insects or vertebrates (e.g. Belyi *et al*., 2010; Crochu *et al*., 2004; Frank and Wolfe, 2009; Geuking *et al*., 2009; Horie *et al*., 2010; Tanne and Sela, 2005; Taylor *et al*., 2010). Among these studies, one clearly correlates this viral integration with virus protection in insects (Maori *et al*., 2007). IAPV does not provoke CCD in all the beehives; some are resistant. Recently, it was shown that individuals that are resistant to viral infection carried integrated fragment of IAPV in their own genome. Interestingly, in contrast to bees whose genome is free of IAPV sequence, those that harbour integrated IAPV are not infected by latent IAPV

and are resistant to IAPV challenge by a yet uncharacterized mechanism (Maori *et al*., 2007). Regardless of the mechanisms involved, it appears that the integration of viral sequence by some eukaryotic genomes may constitute a heritable mechanism of protection against some viral diseases adding a new weapon to the arsenal deployed by insects to fight against viruses.

4 Viral escape from RNAi

As part of the arms race between hosts and pathogens, viruses have evolved mechanisms to minimise or suppress the effect of the host response, which aims at limiting viral replication. Given its major role in antiviral defence in insects, RNAi is not an exception to the rule.

4.1 VIRAL SUPPRESSORS OF RNAI

VSRs are viral-encoded proteins that suppress the effect of RNAi. They were first identified in plants (Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998; Kasschau and Carrington, 1998; Li *et al*., 1999) but several insect viruses such as the DCV, the Cricket Paralysis virus (CrPV) and the FHV also encode VSRs. As the role of VSRs has already been extensively reviewed (e.g. Li and Ding, 2006), only their main features are excerpted below.

Although VSRs share the same function, they interfere with the RNAi machinery at different steps through different mechanisms. For instance, (i) DCV1A (DCV) binds to long dsRNA replication intermediates, thus protecting them from being cleaved by Dcr-2 (van Rij *et al*., 2006), (ii) P19 (plant tombusvirus) sequesters siRNAs thus preventing their loading into AGO2-containing RISC (Silhavy *et al*., 2002), (iii) CrPV1A (CrPV) directly interacts with AGO2 and compromises its activity (Nayak *et al*., 2010) and (iv) P20 and CP of the citrus tristeza virus inhibits the systemic spread of RNAi effects (Lu *et al*., 2004, for a more detailed list of VSRs see Li and Ding, 2006).

Given the major role of dsRNA molecules in RNAi, it is not surprising that most of the VSRs are dsRNA-binding proteins. However, the structures they adopt are not related (Li and Ding, 2006) even though VSRs of viruses belonging to the same family are often present at equivalent genomic locations. Along the same line, the DCV and the CrPV are closely related viruses, as illustrated by the strong similarities between the sequences of their non-structural proteins (71%). However, the sequence and the function of their VSRs are completely unrelated (see above). Together, these observations strongly suggest that the acquisition of VSRs results from convergent evolutionary mechanisms, resulting from selection favouring viruses that escape the effects of RNAi.

Finally, some viruses such as the SINV do not encode any known VSR. Under normal conditions, SINV infection is innocuous for mosquitoes and *Drosophila*. However, when VSRs are expressed from its genome, SINV

becomes pathogenic (FHV B2, Cirimotich *et al*., 2009, DCV1A and CrPV1A, Nayak *et al*., 2010). Importantly, neither of these suppressors provokes such deleterious effects when over-expressed in *Drosophila* (Berry *et al*., 2009; Chou *et al*., 2007; Nayak *et al*., 2010). Altogether these data strongly suggest that: (i) VSRs are *bona fide* virulence factors as they reduce viral pathogenicity when mutated or absent (Galiana-Arnoux *et al*., 2006) but are sufficient to promote pathogenicity in an otherwise innocuous virus; (ii) RNAi reduces viral replication to below a threshold under which the virus is not pathogenic; this threshold is in turn reduced by VSRs and (iii) non-pathogenic viruses such as SINV have an intrinsic pathogenic potential that is revealed when the effects of RNAi are lessened. These conclusions are summarised in Fig. 3.

4.2 VIRAL DIVERSITY

The RNA-dependent RNA polymerases (RdRp) from RNA viruses lack proofreading activity. Consequently, RNA viruses accumulate mutations at high frequency ($\sim 10^{-4}$/replication cycle, Sanjuan *et al*., 2010). Therefore, the genome of viral samples cannot be averaged to its consensus sequence but should be considered as the sum of genomic variants present in the samples (Domingo *et al*., 2006). The role of the accumulation of mutations as a possible RNAi escape mechanism has been indirectly observed in two different systems.

In vertebrate cells the transfection of synthetic siRNA directed against the viral consensus sequence inhibits viral replication while selecting genomic variants that present mutations in the sequence targeted by the siRNA (e.g. Boden *et al*., 2003; Das *et al*., 2004; Gitlin *et al*., 2005); these mutations prevent perfect base-pairing between the siRNA and the viral RNA variants which then escape from degradation by the RNAi machinery. These results showed for the first time that mutations in a viral genome act as a countermeasure to RNAi-mediated degradation. However, these results were obtained *in vitro*, using organisms that naturally do not mount an antiviral RNAi response after infection.

In *Culex pipiens*, oral infection with West Nile virus triggers a natural RNAi response in the midgut of the infected mosquitoes (Brackney *et al*., 2009). Using the sequence of vsiRNAs as a snapshot of the viral genomes being targeted by the RNAi machinery, the authors showed an increase in viral diversity through the course of the infection. This diversification in viral sequence was greater in regions of the West Nile virus genome that included vsiRNAs. In view of these results, the authors proposed that RNAi promotes viral population diversity. This tempting interpretation should be balanced by three main considerations: (i) the low coverage of vsiRNAs along the viral genome may result in a biased representation of genome diversity, especially in the less covered regions; (ii) the increased number of mutations observed in vsiRNAs may reflect an increase in viral diversity, but does not imply a role for RNAi in generating this diversity and (iii) no evidence showed that the vsiRNAs were actually loaded into siRISC, thus reflecting an active sequence-specific targeting of viral genomes.

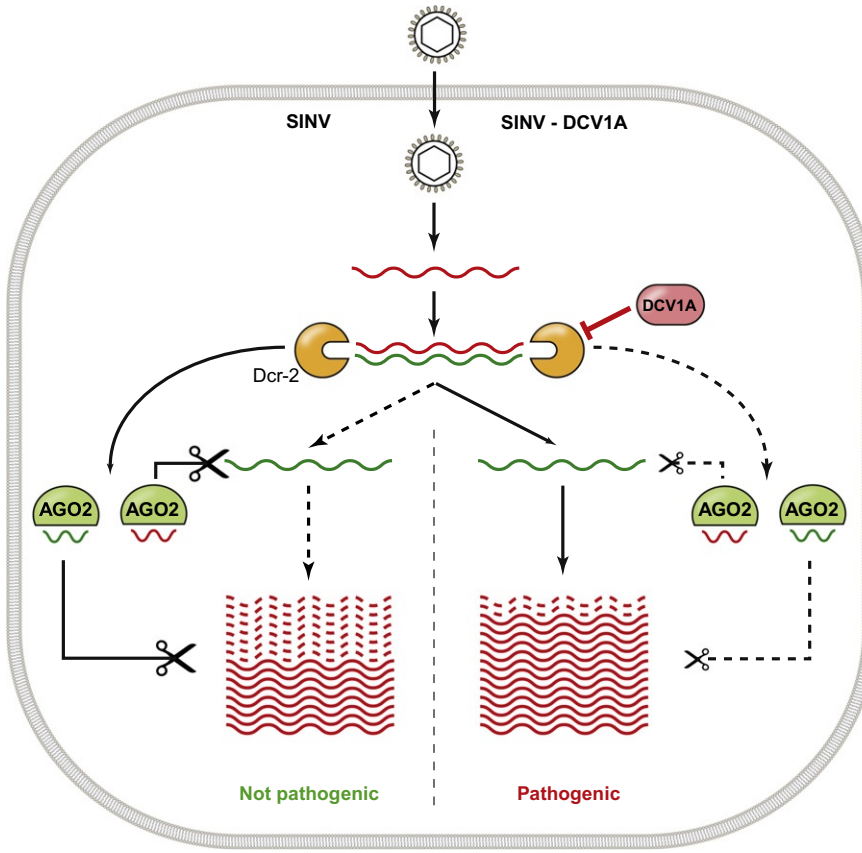

FIG. 3 Model illustrating the impact of VSR on RNAi, viral replication and outcome as exemplified by the SINV (left) and DCV1A-expressing SINV (right). Upon SINV infection (left side), the virus releases its positive strand genome (red) which is first translated and then used as a template for synthesis of the negative strand genome (green). In turn, the negative strand RNA serves as template for the synthesis of numerous positive strand RNAs that will be translated to increase the pool of viral proteins and encapsidated in newly synthesised viruses. Through this replication process, RNAi targets various molecular forms of the viral RNA. First, dsRNA replication intermediates (green and red) are cleaved by Dcr-2 and SINV-derived siRNAs are loaded into AGO2. These siRNAs guide the cleavage of viral RNAs of both polarity, limiting the accumulation of viral RNAs. Under these wild-type conditions, SINV is not pathogenic for insects. (Right side) The expression of the long dsRNA-specific binding protein DCV1A from the SINV genome protects the dsRNA intermediates of replication from degradation by Dcr-2. Consequently, fewer siRNAs are produced and loaded into AGO2, leading to a greater accumulation of viral RNAs. Under these conditions, DCV1A-expressing SINV is pathogenic for *Drosophila* contrary to the wild-type virus.

Nevertheless, data collected from both systems suggest a potential role of mutations in the viral genome as an escape mechanism from RNAi. Further experiments are necessary to directly assess the relationship between viral diversity and RNAi *in vivo*.

4.3 OTHER ESCAPE MECHANISMS

The relationship between RNAi and viral replication has only been studied at the molecular level regardless of the intracellular physiology of this interaction. Nevertheless, several aspects of viral replication may protect viral RNA from being degraded by the RNAi machinery. First, viral replication takes place at the membrane of virus-induced cellular compartments of various origins that may limit the accessibility of Dcr-2 to the dsRNA. Consequently, the antiviral activity of Dcr-2 would be limited to the subset of replication intermediates that escape from this protective niche. Interestingly, the site of replication may also influence the virus-RNAi relationship. The FHV replication complex is localised at the mitochondrial outer membrane in different hosts (Miller and Ahlquist, 2002; Miller *et al*., 2001). In yeast, the retargeting of the FHV replisome to the endoplasmic reticulum results in increased RNA synthesis (Miller *et al*., 2003) through an uncharacterised mechanism. This increased RNA synthesis could result from more efficient replication or better protection against degradation. These hypotheses remain to be investigated in light of antiviral RNAi in an organism, which contrary to *Saccharomyces cerevisiae*, possesses proficient RNAi machinery. Finally, translation and encapsidation may provide shelter for viral RNA by limiting their accessibility to AGO2.

5 Closing remarks

In the past decade, RNAi has been identified as a major mechanism of gene regulation with impacts on several cellular processes. In immunity, RNAi pathways play central roles in immune reactions both as regulators and as effectors. In insects, RNAi seems to be predominantly involved in antiviral defence through its direct antiviral activity; in vertebrates, RNAi has mostly been implicated in the regulation of immune responses. This last function for RNAi has not been addressed in insects but results obtained in other organisms suggest that such a role is conserved. Interestingly, while antiviral RNAi as defined in insects remains to be established in vertebrates, the intracellular recognition of dsRNA as a signature of viral infection is shared by both (Deddouche *et al*., 2008). This is reminiscent of the antimicrobial response where the intracellular pathways are conserved between insects and vertebrates, while the upstream activation and the downstream effectors are different.

One of the most intriguing aspects of the antiviral RNAi in insects is its inability to eliminate the virus from the organism; in fact RNAi controls but

never clears a viral infection. Whether controlling (functional efficiency) over eliminating (molecular efficiency) the virus is the ideal, rather than a compromise, for the insect is subject of debate. To address this question, let us consider the case of the SINV. Wild-type SINV is not pathogenic for insects in a siRNA pathway-dependent fashion. Therefore, the siRNA pathway is functionally efficient and controlling viral replication is sufficient to limit the deleterious effects of a viral infection. Nevertheless, the question of molecular efficiency remains unanswered. Several reasons may account for such an 'ineffectiveness'. First, it has been observed that genes encoding some components of the siRNA pathway display a more rapid evolutionary rate that non-immune genes presumably due to their targeting by viral suppressing factors. Therefore, the fast evolution of these genes prevents the siRNA pathway from becoming optimal in eradicating the virus while adapting to viral countermeasures. Second, it has been recently shown that the intracellular bacterium *Wolbachia* protects *Drosophila* from viral infection (Teixeira *et al.*, 2008) most likely in an RNAi-independent fashion. Moreover, it was suggested that the prevalence of *Wolbachia* infection in natural populations of *Drosophila* confers a selective advantage, presumably against infection by RNA viruses (Hedges *et al.*, 2008). This additional layer of defence may relax selection for a more molecularly efficient siRNA pathway by limiting viral replication upstream of or in parallel to it. Finally, some aspects of viral physiology such as encapsidation may render the clearance of the virus impossible. While most of the work has focused on the molecular players involved in host-virus interaction, further experiments are needed to evaluate the relationships between viral replication and RNAi in their physiological context.

Along the same lines, several aspects of RNAi biology remain to be addressed in insects including the following: (i) do miRNAs regulate immune responses, (ii) is there a role for RNAi in response to DNA viruses, (iii) what are the spatial and temporal relationships between viral replication and RNAi processing and (iv) can bacterial or fungal pathogens turn RNAi to their own benefit as observed for some viruses. Addressing these questions would provide a better understanding of the implication of small RNA pathways as surveillance, signalling and effector mechanisms for immunity.

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Note added in proof

Due to the extensive time between writing and peer-review of the manuscript, its acceptance for publication (January 2011) and its final publication, some of the sections are not up to date. For example, we have recently demonstrated the production of virus-derived piRNAs following arbovirus infection in three mosquito cells lines: C6/36 and U4.4 from *A. albopictus*, and Aag2 from *A. aegypti* (Vodovar *et al*., PlosOne, 2012, in press).

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