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# The Interplay Between Viruses and RNAi Pathways in Insects

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## Keywords

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## Abstract

As an overarching immune mechanism, RNA interference (RNAi) displays pathogen specificity and memory via different pathways. The small interfering RNA (siRNA) pathway is the primary antiviral defense mechanism against RNA viruses of insects and plays a lesser role in defense against DNA viruses. Reflecting the pivotal role of the siRNA pathway in virus selection, different virus families have independently evolved unique strategies to counter this host response, including protein-mediated, decoy RNA–based, and microRNA-based strategies. In this review, we outline the interplay between insect viruses and the different pathways of the RNAi antiviral response; describe practical application of these interactions for improved expression systems and for pest and disease management; and highlight research avenues for advancement of the field.

## INTRODUCTION

Like all organisms, insects are subject to infection by viruses, but only with the advent of next-generation sequencing have the abundance and diversity of insect-associated viruses been revealed (7, 63). The course and outcomes of viral infections rely on complex molecular host–virus interactions. Viruses hijack host cellular factors for infection and replication, while the host counters by restricting access to key factors and/or by mounting an antiviral immune response. A major player in the invertebrate antiviral immune response is the highly conserved small interfering RNA (siRNA) pathway of the RNA interference (RNAi) response (117). The siRNA pathway is the primary antiviral defense mechanism against insect viruses with RNA genomes (86) but also functions in defense against insect viruses with DNA genomes. Virus-derived double-stranded RNA (dsRNA) triggers the antiviral siRNA pathway in host cells, which can completely block virus replication.

Study of the interplay between viruses and the siRNA pathway has resulted in several tools, including methods for detection of covert viruses in insect cell lines (13, 14), optimized baculovirus expression systems (17, 101), and the potential use of virus-induced gene silencing (VIGS) for pest and disease management (106). The (re)emergence of insect vector-borne viruses such as dengue, Chikungunya, and Zika viruses highlights the importance of understanding insect–virus molecular interactions for providing solutions for global health.

**siRNA pathway:** small interfering RNAs of approximately 20–22 base pairs processed from long dsRNA by Dicer-2; this pathway is mainly antiviral

**RNA interference (RNAi):** comprised of three pathways; mechanism for sequence-specific gene, virus, or transposon silencing based on sRNA production

**Virus-induced gene silencing (VIGS):** use of a virus vector to deliver silencing RNAs to silence a target gene

**piRNA pathway:** PIWI-interacting RNAs of approximately 26–32 nucleotides that primarily function to silence transposons in the germline

**Dicer-2 (Dcr2):** RNase III nuclease that cleaves dsRNA into siRNAs

**Argonaute-2 (Ago2):** primary RISC component of the siRNA pathway mediating target recognition and cleavage of viral genome and transcripts

## INSECT RNAi-DEPENDENT IMMUNE MECHANISMS AGAINST VIRAL INFECTIONS

The three primary small RNA (sRNA)-directed RNA silencing pathways in insects are the siRNA, microRNA (miRNA), and PIWI-interacting RNA (piRNA) pathways (139). While all three pathways center around the silencing of target RNA by Argonaute family proteins, which themselves are directed by the sRNAs in a sequence-specific manner, they are differentiated by the length and origin of the guiding sRNAs, enzymatic requirements, and functional outcomes.

### The siRNA Pathway

RNAi directed by siRNAs is a potent antiviral mechanism against both RNA and DNA viruses in a wide variety of eukaryotic organisms. This pathway begins with the recognition of dsRNA of viral origin in the cytoplasm by the RNase-III enzyme Dicer-2 (Dcr2) (5) (**Figure 1**). dsRNA is then cleaved by Dcr2 into approximately 21-nucleotide (nt) siRNA duplexes (109) containing 19 base-paired nts flanked by 2-nt 3′ overhangs (23). One strand of the siRNA duplex, known as the guide strand, is incorporated into the RNA-induced silencing complex (RISC), while the other strand (the passenger strand) is degraded (72, 105). The guide strand is subsequently 2′-*O*-methylated at the 3′ end by DmHen1 methyltransferase to form a mature RISC (28).

Argonaute-2 (Ago2) is the catalytic core of the RISC. Ago2 stably binds the guide strand within the protein complex through interactions between the 3′ and 5′ ends of the guide strand and the Ago2 PAZ and MID domains, respectively (15). siRNAs bound to Ago2 guide the RISC to complementary RNAs in the cytoplasm in a sequence-specific manner through Watson-Crick base pairing. Following target recognition, Ago2 catalyzes cleavage of target RNAs between nts 10 and 11 as measured from the 5′ end of the guiding siRNA (24, 104). RNA molecules cleaved by Ago2 are then rapidly degraded through the activity of the XRN1 exoribonuclease and the exosome (94). Cleavage by Ago2 is sequence specific and requires perfect base pairing between nts 2–21 of the guide strand (70).

Single-stranded RNA (ssRNA), dsRNA, single-stranded DNA, and double-stranded DNA viruses are all targeted by siRNAs in insects (9, 85, 97, 128, 138). During the infection cycles

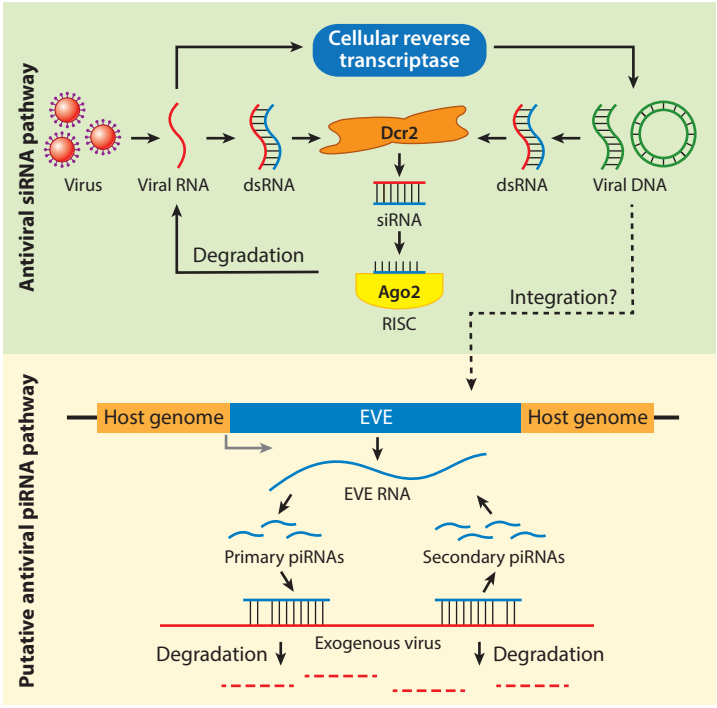


**Figure 1**

The antiviral small RNA pathways. While the antiviral role of the small interfering RNA (siRNA) pathway is well established, direct evidence for a role of the PIWI-interacting RNA (piRNA) pathway in antiviral immunity is lacking. We propose that the interaction of endogenous viral elements (EVEs) with exogenous cognate viruses could generate viral piRNAs with an antiviral role. These EVEs could originate from the integration of viral DNA forms of RNA viruses produced during viral infection in insects. In this way, there may be crosstalk between the two pathways to maintain host fitness during viral infection.

of RNA viruses, dsRNA is formed from the genomes of dsRNA viruses or by replication intermediates of ssRNA viruses (113). DNA viruses are thought to produce dsRNA by convergent transcription. For both DNA and RNA viruses, secondary structure within single-stranded viral RNA or transcripts can serve as a source of dsRNA (113). Notably, siRNAs can also be produced from endogenous sources of dsRNA (endo-siRNAs), and these are implicated in gene regulation and repression of transposable elements (TEs) (33).

Interaction between viral RNA and the siRNA pathway is indicated by the Dcr2-dependent accumulation of approximately 21-nt virus-derived siRNAs during virus infection in insects. The antiviral nature of the siRNA pathway is clearly demonstrated by the finding that knockout of Dcr2 or Ago2 results in enhanced virus replication and increased host mortality in a variety of systems (1, 52, 110, 128). This is further supported by the observation that both DNA and RNA viruses encode suppressors of siRNA-based RNAi (detailed below).

## The miRNA Pathway

Unlike siRNAs, which are usually produced from exogenous sources of dsRNA, the precursors of miRNAs are transcribed from endogenous loci by RNA polymerase II (63). These transcripts, known as primary miRNAs, are highly structured molecules that are cleaved by Drosha into approximately 60-nt hairpins known as precursor miRNAs (62). Precursor miRNAs are exported

to the cytoplasm, where they are recognized and cleaved by Dicer-1 to form a miRNA duplex (64, 69). One strand of the miRNA duplex is then loaded onto Argonaute-1 (Ago1) within the miRNA induced silencing complex (28). Mature miRNAs are approximately 22 nt, and in insects, they typically direct silencing of endogenous target RNAs through imperfect base pairing (3). Like Ago2, the endonuclease activity of Ago1 requires perfect base pairing along the guide miRNA (3). Because miRNA target recognition relies on imperfect base pairing, miRNA-directed RNA silencing usually involves translational repression through Ago1-mediated deadenylation and decapping (48). Relatively little work has explored the antiviral potential of miRNAs in insects. In mammals, miRNAs are known to exert antiviral effects by directing transcriptional changes that result in an unfavorable state for virus replication. More infrequently, mammalian miRNAs can directly target viral RNA (19). While virus infection in insects is known to alter the abundance of some host miRNAs in a temporal and tissue-specific manner, the effects that such changes might have on virus replication are unclear (81).

## The piRNA Pathway

The most recently discovered sRNA-based silencing pathway, the piRNA pathway, is the primary mechanism of TE repression in the animal germline (20). Insect piRNA pathway details are most clearly understood in *Drosophila melanogaster*, but the general mechanisms of piRNA biogenesis are conserved in most animals (30). piRNA biogenesis is classically divided into the primary and secondary pathways (7). In the *D. melanogaster* primary piRNA pathway, discrete genomic loci (piRNA clusters) give rise to long piRNA precursor transcripts, which are exported to the cytoplasm and fragmented by Zucchini endonuclease (Zuc) into precursor piRNAs (7, 91). Precursor piRNAs are then loaded onto Piwi or Aubergine (Aub), and their 3′ ends are trimmed and 2′-*O*-methylated, resulting in 26–32-nt mature primary piRNAs (28, 41). Because Zuc cleavage preferentially occurs just upstream from uracil residues, and because the MID domains of PIWI-clade Argonaute proteins favor interactions with uracil residues, primary piRNAs have a strong bias for uracil as the fifth nt (1U bias) (18, 91). piRNA clusters contain a high density of TE-derived sequences (7). Thus, the primary piRNA pool contains an abundance of piRNAs specific to TEs.

The secondary piRNA pathway, also known as the ping-pong cycle, involves recognition of complementary RNA by Aub-bound primary piRNAs via nearly perfect Watson-Crick base pairing (7, 44). Like Ago2, Aub catalyzes the cleavage of target RNA between nts 10 and 11 with respect to the 5′ end of the guiding primary piRNA (7). Cleaved target RNA with a 5′ end defined by Aub cleavage is then loaded onto Argonaute-3 (Ago3) and processed into a 26–32-nt secondary piRNA with a 2′-*O*-methylated 3′ end defined by Zuc cleavage or exonucleolytic degradation of cleaved RNA by nibbler (7, 36, 39, 41, 78, 132). Secondary piRNAs have a strong bias for adenine as the 10th nt (10A bias) and are antisense to the primary piRNA that directed the cleavage event resulting in their formation (7). Ago3-bound secondary piRNAs direct cleavage of complementary RNA targets, which are subsequently processed into Aub- or Piwi-bound primary piRNAs (7). Thus, the ping-pong cycle is a series of reciprocal cleavage events that serves as a post-transcriptional silencing mechanism and amplifies the piRNA-based response against active TEs (7, 20). piRNAs are distinguished from other types of sRNAs by their size (26–32 nt), and ping-pong-dependent piRNAs can be identified by 1U and 10A biases, as well as by a distance of 10 nt between the 5′ ends of complementary piRNAs.

Interestingly, virus-derived, ping-pong-dependent piRNAs (vpiRNAs) are present during infection of *Aedes aegypti* and *Aedes albopictus* mosquitoes with several different ssRNA viruses (77), as well as in cell lines derived from these species. vpiRNAs derived from a single-stranded DNA virus were also observed during infection of *A. aegypti*–derived Aag2 cells (97). Unlike *D. melanogaster*, which expresses only three PIWI-clade Argonaute proteins (Aub, Ago3, and Piwi), *A. aegypti* and

*A. albopictus* express eight PIWI-clade Argonaute proteins (Ago3 and Piwi1–7) (12, 65). In Aag2 cells, Piwi5 and Ago3, but none of the other PIWI-clade Argonaute proteins, are required for biogenesis of vpiRNAs (76). In contrast, production of TE-derived piRNAs relies on a more diverse set of PIWI proteins (76). This suggests that the novel PIWI-clade Argonaute proteins in mosquitoes have undergone functional diversification to produce piRNAs from a variety of RNA sources. vpiRNAs are not produced during RNA or DNA virus infection in *D. melanogaster* and have not yet been observed outside of mosquitoes (98).

Despite their essential roles for vpiRNA biogenesis, knockdown of Piwi5 and Ago3 does not lead to enhanced virus replication in Aag2 cells (76, 111, 124, 129). Knockdown of Piwi4 does not significantly impact vpiRNA abundance in Aag2 cells or *A. aegypti* mosquitoes but does lead to increased replication of several RNA viruses in Aag2 cells, as well as increased replication of dengue virus in vivo in *A. aegypti* (76, 111, 124, 129, 130). While intriguing, the links among PIWI protein expression, vpiRNA abundance, and virus replication in mosquitoes are not yet clear, and direct evidence of an antiviral role for the piRNA pathway is lacking.

**Immune priming:** memory in insect immune response to a subsequent exposure to a pathogen

**Endogenous viral element (EVE):** complete or partial viral sequence integrated into host genome

## Immune Memory

A characteristic feature of the antiviral response in jawed vertebrates is the maintenance of long-lasting protection against future infections with the same virus (immune memory). Because insects lack antibodies, they have generally been considered incapable of preserving a functional record of prior infections. However, this view has been challenged by recent results demonstrating that DNA forms of RNA virus sequences are produced during viral infections, serve as sources of siRNAs, and are maintained even after clearance of the infection (34, 35, 79, 100, 123, 124). We are only beginning to understand the mechanisms underlying this immune memory or immune priming in insects.

The reverse transcriptases encoded by endogenous retrotransposons can reverse transcribe viral RNA in insects, resulting in the formation of chimeric viral DNAs (vDNAs) consisting partly of virus sequence and partly of TE sequence (34, 35, 100). Importantly, vDNAs are transcribed and serve as a source of the siRNAs that modulate virus infection (34, 35, 100, 123) (**Figure 1**). The prevention of vDNA formation during virus infection of *D. melanogaster* or *A. albopictus* decreases virus-specific siRNA abundance and increases insect mortality (34, 35). Remarkably, vDNAs generated from RNA viruses taken up by hemocytes in *D. melanogaster* give rise to virus-specific siRNAs that are secreted in exosome-like vesicles capable of priming an antiviral response in uninfected cells (123). Similarly, vDNAs purified from *D. melanogaster*–derived S2 cells infected with *Flock house virus* (FHV) confer virus-specific protective immunity following injection into naïve *D. melanogaster* flies, raising the possibility that vDNA may serve as a biologically relevant source of long-lasting and sequence-specific nucleic acid–based memory (100). Most studies of insect antiviral mechanisms have employed intrathoracic injection of virions, which generally results in death of the host or the development of a lifelong persistent infection (79). The persistent state in *D. melanogaster* and *A. albopictus* requires the formation of vDNA, highlighting its importance for long-term immune processes (34, 35). Because infections initiated by intrathoracic injection are not typically cleared, the potential role of vDNA in priming a protective response against future infections is difficult to assess.

vDNAs are episomal molecules produced during the course of virus infection (100). In contrast, the sequences of endogenous viral elements (EVEs) derive from full or partial integrations of viral sequence into the host genome (43, 51). EVEs from both DNA and RNA viruses have been described in a wide variety of eukaryotes (43, 51, 125). Nonretroviral RNA virus sequences within host genomes are surprising because these viruses do not undergo a DNA stage during replication.

While the mechanisms of EVE generation are unclear, it is tempting to speculate that vDNAs are precursors of EVEs.

**Viral suppressor of RNAi (VSR):** viral mechanism for countering host siRNA-mediated antiviral response

There are several well-known examples of EVEs being co-opted and playing important roles in the biology of the host (27, 75). Since their discovery, EVEs have been speculated to play an antiviral role (58) (**Figure 1**). In support of their antiviral potential, many EVEs are transcribed and contain uninterrupted open reading frames (ORFs). However, few reports have presented experimental evidence (29, 121). In honey bees, 30% of wild populations harbored an EVE derived from *Israeli acute paralysis virus* (IAPV) encoding a protein capable of cross-reacting with an antibody specific for the corresponding IAPV capsid protein (71). In vivo, individuals carrying this EVE exhibited reduced mortality following injection of IAPV compared to individuals without the EVE.

Given that nucleic acid–based defense mechanisms represent the primary antiviral strategy in arthropods, and that vDNA is capable of inducing immune priming via siRNAs, it is hypothesized that EVEs might similarly provide the specificity determinants of a long-lasting and heritable nucleic acid–based silencing system. In support of this, EVEs in *A. aegypti* and *A. albopictus* were found to be enriched within piRNA clusters and give rise to piRNAs (95, 135) (**Figure 1**). This finding was extended to arthropods in general by an analysis of the EVEs present within arthropods with sequenced genomes that have corresponding publicly available sRNA data sets (125).

So far, experiments on the antiviral potential of EVE-derived piRNAs have been limited to Aag2 cells (124, 135). An EVE derived from *Phasi Charoen-like virus* (PCLV) gives rise to a single piRNA mapping antisense to the PCLV genome (135). In Aag2 cells persistently infected with PCLV, the 5′ end of this piRNA was separated by 10 nt from a piRNA mapping to the opposite strand, and knockdown of Piwi4 led to an approximately twofold increase in PCLV RNA levels (135). The Aag2 genome also contains EVEs derived from *Cell fusing agent virus* (CFAV) (124, 135). Aag2 cells persistently infected with CFAV show increased CFAV RNA levels, and knockdown of Piwi4 or Ago3 reduced CFAV-derived piRNA abundance (124). Although this evidence supports an antiviral role for these proteins, it is difficult to confirm an antiviral role for EVE-derived piRNAs based on CFAV-derived piRNA abundance without more detailed sRNA mapping profiles. Intriguingly, insertion of sequences corresponding to piRNA-producing EVEs within the *Sindbis virus* 3′ UTR leads to diminished replication of the recombinant viruses in Aag2 cells following Piwi4 knockdown (124). The sRNA profiles during infection with such recombinant viruses should be the subject of future investigation.

## VIRAL COUNTER-DEFENSE MECHANISMS

### Viral Suppressors of siRNA-Based RNAi

Both RNA and DNA viruses encode viral suppressors of RNAi (VSRs) to counter the negative impact of the siRNA pathway on virus replication. VSRs from different virus families lack sequence or structural similarity, indicating independent evolution of different mechanisms to counter the siRNA pathway. VSRs act via four primary mechanisms: (*a*) binding to long dsRNA to prevent Dcr2-mediated cleavage, (*b*) binding to siRNA to prevent siRNA loading into RISC, (*c*) interfering with Dcr2 or Ago2 function, and (*d*) degrading siRNAs. Some VSRs combine multiple modes of action (**Table 1**).

The VSRs of viruses that infect *D. melanogaster* are particularly well studied. While homologous at the genetic level, the two dicistroviruses *Cricket paralysis virus* (CrPV) and *Drosophila C virus* (DCV) exhibit distinct virulence characteristics in wild-type *Drosophila*. CrPV results in paralytic, lethal (acute) infection, while DCV causes persistent, nonlethal (chronic) infection. The VSRs of CrPV and DCV map to the 1A protein at the N terminus of the ORF1 polypeptide but have low sequence similarity (approximately 9%) and lack homology to other proteins (89, 128, 133).

**Table 1 VSRs encoded by insect viruses and arboviruses**

| Family | Virus | VSR | Binds to | | | Inhibits | | Degrades siRNA | Decoy RNA | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | dsRNA | siRNA | Dcr2 | Ago2 | Dcr2 | | | |
| **RNA virus** | | | | | | | | | | |
| *Nodaviridae* | *Flock house virus* | B2 | X | X | X | – | | | | 2, 16, 66 |
| | *Nodamura virus* | B2 | X | X | – | | X | | | 120 |
| | *Wuhan nodavirus* | B2 | X | X | X | | | | | 102, 103 |
| | *Mosinovirus* | B2 | X | – | | | | | | 114 |
| *Dicistroviridae* | *Drosophila C virus* | 1A | X | – | | – | | | | 128 |
| | *Cricket paralysis virus* | 1A[a] | – | – | | X | | | | 90, 134 |
| *Birnaviridae* | *Drosophila X virus* | VP3 | X | X | | – | | | | 126 |
| | *Culex Y virus* | VP3 | X | X | | – | | | | 126 |
| Unassigned | *Drosophila melanogaster Nora virus* | VP1 | – | – | | X | | | | 127 |
| | *Drosophila immigrans* Nora-like virus | VP1 | | | | X | | | | 127 |
| *Flaviviridae* | *Dengue virus* | sfRNA | | | | | | | X[b] | 112 |
| | *West Nile virus* | sfRNA | | | | | | | X[c] | 112 |
| **DNA virus** | | | | | | | | | | |
| *Ascoviridae* | *Heliothis virescens ascovirus* | RNase III | | | | | | X | | 45 |
| *Iridoviridae* | *Invertebrate iridescent virus* | IIV6–340R | X | X | | – | | | | 8 |

[a]Also disrupts stress granule formation (56).
[b]Based on use of human Dicer. Inhibition of Dicer-2 in insects is assumed.
[c]Inhibits siRNA-based RNA interference in insect cells. This function presumed based on similarity to *West Nile virus* sfRNA.
Abbreviations: Ago2, Argonaute-2; Dcr2, Dicer-2; dsRNA, double-stranded RNA; sfRNA, subgenomic flavivirus RNA; siRNA, small interfering RNA; VSR, viral suppressor of RNA interference.

Expression of CrPV 1A or DCV 1A in *Drosophila* S2 cells blocked the siRNA pathway, increasing cell susceptibility to virus infection (89, 128). The pathogenicity traits of CrPV and DCV are reflected in the different efficacies of their respective VSRs in suppressing the siRNA pathway: The 166-amino-acid CrPV 1A prevents the Ago2 RISC from targeting RNA (134), while the 97-amino-acid DCV 1A binds to dsRNA, preventing processing by Dcr2 (128). Introduction of CrPV 1A into a recombinant *Sindbis virus* markedly increased virulence, while introduction of DCV 1A resulted in only a modest increase in virulence (89). Two elements within CrPV 1A that bind specific host proteins confer dual functionality in suppression of the siRNA pathway. In addition to acting as a competitive inhibitor to block Ago2, a BC box domain mediates ubiquitination of Ago2, thereby targeting Ago2 for degradation (90).

Stress granules form in the cytoplasm following inhibition of translation. They appear to function as a cellular antiviral response by sequestering viral proteins or RNA. Some viruses have evolved measures to counter stress granule formation. For example, CrPV and DCV, which inhibit S2 cell translation, do not result in formation of stress granules. CrPV 1A disrupts stress granule formation by a mechanism independent of its VSR function (56).

The 106-amino-acid B2 protein of FHV is a VSR that binds to dsRNA and siRNAs, inhibiting the siRNA pathway by sequestering siRNAs and preventing processing of dsRNA (2, 16, 66). B2 also binds to dsRNA regions in FHV RNA2 to prevent movement into cytoplasmic granules (which differ from stress granules) where translation is silenced. Deletion of B2 thus impairs translation of the capsid protein from RNA2 (99). FHV B2 is arguably the best studied of the insect virus VSRs due to the early availability of an infectious replicon that allows for strong genetic evidence. Indeed, other insect VSRs have been tested for their ability to rescue the FHV ΔB2 replicon (25). Transgenic *Drosophila* (6) show that, in addition to blocking antiviral defense, B2 and CrPV 1A also interfere with transposon silencing in somatic tissues by endo-siRNAs.

The Nora virus VP1 and Nora-like virus VP1 proteins function as VSRs by interacting with Ago2 and blocking cleavage of target sequences (127). As VSRs provide counter-defense against host antiviral RNAi, both VSRs and their targets in the siRNA pathway (Dcr2, R2D2, and Ago2) evolve rapidly (93). This rapid evolution can result in VSRs that are host specific. For example, the Nora-like virus VP1 binds *Drosophila immigrans* Ago2 but not *D. melanogaster* Ago2 (127). This observation highlights the importance of studying VSR activity in a relevant host species, rather than using a more tractable model species.

In addition to serving as hosts for mosquito-specific viruses, some mosquitoes also vector viruses (arthropod-borne viruses, or arboviruses) of medical and veterinary importance. VSRs have been identified from arboviruses in the genera *Alphanodavirus* (*Nodaviridae*) (120) and *Flavivirus* (*Flaviridae*) (50). *Nodamura virus* (*Nodaviridae*) is a mosquito-transmitted arbovirus that, similar to FHV, encodes a B2 VSR protein that binds dsRNA and siRNAs (120). *Dengue virus* nonstructural protein 4B (NS4B) is reported to function as a VSR, although the mechanism is unclear (50).

Three VSRs have been identified in insect DNA viruses. *Heliothis virescens ascovirus* encodes an RNase III that degrades siRNAs (45). *Invertebrate iridescent virus 6* (IIV-6) encodes a VSR, 340R, that binds siRNAs and dsRNA. This VSR rescues FHV lacking its VSR, confirming the ability of this protein to antagonize antiviral RNAi (8). IIV-6 completely masks the impact of the siRNA pathway in *Drosophila* (10). The inhibitor of apoptosis, P35, derived from the baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) also functions as a VSR acting downstream of cleavage by Dcr2 (74).

## Decoy RNAs

*West Nile virus* (WNV) and *Dengue virus* are mosquito-transmitted flaviviruses. These viruses produce noncoding subgenomic flavivirus RNAs (sfRNAs) as a result of incomplete degradation of

genomic RNA (112). The stem-loop structures of these sfRNAs may act as competitors or decoys for Dcr2 activity, thereby decreasing the efficiency of the antiviral RNAi response. Similarly, hot spots in the genome that were more frequently targeted were identified based on siRNA profiles of two mosquito cell lines following infection with the alphavirus *Semliki Forest virus*. These areas may serve as decoy RNA sequences, reducing the efficacy of the antiviral response by providing regions that are more susceptible to siRNA-mediated RNAi based on structure, but less damaging to the replicating virus (118). This phenomenon was also noted for the phlebovirus *Rift Valley fever virus*, whose defective interfering particle and S segment were heavily targeted for siRNA production but were not stabilized for Ago2 loading (108). This putative decoy strategy represents an alternative mechanism for evasion of RNAi-based antiviral defense.

### Viral miRNA-Based Manipulation of the Host Genome

miRNAs can also influence the outcome of virus infection (82). Some viruses encode miRNAs that target host or viral mRNA with proviral consequences. Viral miRNAs that target viral mRNA can modulate the timing of viral replication or gene expression. For example, *Heliothis virescens ascovirus* miR-1 reduces viral replication, thus delaying viral replication and promoting long-term virus production (46). Similarly, AcMNPV miR-1 targets multiple viral proteins, including ODV-E25, to reduce budded virus production (140, 141), and *Bombyx mori nucleopolyhedrovirus* (BmNPV) miR-3 reduces late gene expression to avoid early detection by the host (115).

Viral miRNAs that regulate host gene function include WNV kun-miR-1, which targets and upregulates *GATA4* to the benefit of the virus (47). BmNPV miR-1 impacts miRNA biogenesis, resulting in increased virus replication by targeting the host gene *Ran* that functions in export of pre-miRNAs from the nucleus into the cytoplasm (116). The reduced miRNAs seen in *D. melanogaster* infection with DCV could result from a similar impact on miRNA biogenesis (83).

## PRACTICAL APPLICATIONS AND IMPLICATIONS

### Practical Applications for Viral Suppressors of RNA

Knowledge of molecular factors contributing to insect virus–RNAi interactions provide opportunities for novel applications. VSRs such as CrPV 1A and FHV B2 can be used ectopically for suppression of the siRNA pathway to reveal latent infections in cell lines (13, 14) or to promote replication of other RNA or DNA viruses (57). VSRs also provide tools to investigate the molecular mechanisms of antiviral RNAi: A single-molecule approach has been employed to elucidate how VSRs discriminate between viral and cellular RNA (25). VSRs can also be used to track dsRNA in live cells (84).

### RNAi for Optimization of Baculovirus Expression

RNAi can be used to optimize protein expression from insect virus–based vectors such as the baculovirus expression system (17, 101). For example, insect cell lines stably transformed to downregulate *caspase-1* by siRNA-based RNAi had prolonged resistance to apoptosis following baculovirus infection, thereby increasing the duration of protein expression (131). Similarly, siRNA-based RNAi has been used to restrict baculovirus gp64 production, reducing the number of baculovirus budded virus contaminants present in candidate vaccines (61), and to improve glycosylation of baculovirus-expressed therapeutic proteins (87). Although investigation of insect virus–derived VSRs is relatively young (42), the potential value of VSRs for improved baculovirus expression of recombinant proteins was demonstrated by use of plant virus–derived VSRs (21, 67).

## Virus-Induced Gene Silencing

The efficacy of dsRNA for gene silencing in insect pests can be severely restricted by nucleases in the saliva or gut that degrade dsRNA. As the sRNA viruses of insects have evolved to overcome this enzymatic challenge, the viral particle could have potential use as an enzyme-resistant dsRNA delivery system. VIGS is the delivery of silencing RNAs from a virus. The use of insect virus–based VIGS vectors in pest management is in its infancy, largely due to the limited number of infectious clones of insect RNA viruses. However, multiple plant virus–based VIGS vectors have been developed, and some have been employed to silence essential genes in crop pests (106). In this system, the plant virus produces silencing RNAs in the host plant that are ingested by insects feeding on the plant, so-called host-induced gene silencing. For example, on infection of *Nicotiana benthamiana*, a *Tobacco rattle virus* VIGS vector produced silencing RNAs targeting three *Manduca sexta* gut cytochrome P450 genes (59). A *Citrus tristeza virus* vector was used to target the abnormal wing disc gene of the Asian citrus psyllid, *Diaphorina citri* (38); a *Tobacco mosaic virus* vector was used to target genes in the potato psyllid, *Bactericera cockerelli*, and citrus mealybug, *Planococcus citri* (55, 136); and a *Potato virus X* vector was used to silence genes in the cotton mealybug, *Phenacoccus solenopsis* (54).

# NEEDS FOR FUTURE RESEARCH

## Natural Pathosystems

Studies of antiviral mechanisms in insects have traditionally relied on a small number of model insects such as *D. melanogaster* and *A. aegypti*. These investigations have often focused on viruses with direct relevance to human health or agriculture, and in many cases, the viruses under study are not natural pathogens of the experimental host. Furthermore, most studies have delivered viruses via intrathoracic injection, bypassing the hosts' natural barriers to viral infection. While these approaches have revealed a wealth of knowledge regarding host–virus interactions, they provide an incomplete and biased picture. Indeed, several recent studies have highlighted the importance of the route of infection for the outcome of infection (4, 37, 73, 79). Different routes of infection are also known to trigger different immune responses. For example, the Toll pathway is required for resistance to oral infection with DCV, CrPV, or FHV in *D. melanogaster* but does not appear to play a role during systemic infections (26). The observation that *D. melanogaster* flies can clear DCV infection independent of RNAi underscores the fact that different antiviral mechanisms are required to control virus infection following different routes of exposure (79). More work is needed to understand both the antiviral responses triggered by different routes of infection and the mechanisms by which these responses restrict virus replication.

Natural host–pathogen systems are shaped by coevolution, and natural pathogens can be subject to more structured and effective immune responses (26, 73). Susceptibility to virus infection also varies among different strains of the same insect species; thus, results obtained from common laboratory strains may not necessarily reflect the situation in nature (96). Such differences highlight the importance of studying host–virus interactions in a more natural context to provide more robust and relevant biological data.

## Antiviral RNAi: Inducible or Constitutive?

A core feature of immune mechanisms is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors, resulting in the induction of innate immune responses. Several cell signaling pathways, including the JAK-STAT, Toll, and Imd pathways, are induced upon pathogen recognition as part of the insect immune system. dsRNA is known

to serve as a potent PAMP in insects, and administration of dsRNA, regardless of the sequence, induces the activation of an antiviral response (11, 22, 68). In some cases, recognition of dsRNA has been attributed to the DExD/H-box helicase of Dcr2, suggesting a possible link between components of the siRNA pathway and non-RNAi-based antiviral responses (22). Despite the central role of RNAi in controlling virus infections in insects, relatively little is known about the regulation of the core components of RNAi. The expression of Dcr2 and Ago2 mRNAs was recently shown to be upregulated in a sequence-independent manner by dsRNA in a variety of systems, and a similar increase in Ago2 and Dcr2 expression was observed upon viral infection in *B. terrestris*, *Apis mellifera*, and *D. melanogaster* (31, 32, 40, 68, 92, 137). Intriguingly, injection of heat-inactivated Zika virus also upregulates the expression of RNAi components in *D. melanogaster* (40). Together, these results suggest that virus presence may be sensed by insects independent of viral replication, and that RNAi is regulated as part of a response that is not virus specific. *D. melanogaster* flies constitutively expressing an active form of dFOXO exhibit elevated expression of Dcr2 and Ago2, establishing a link between stress response signaling and RNAi and providing further support for the inducible nature of RNAi (119). Despite these advances, our understanding of RNAi regulation remains limited. Future studies should address whether the route of infection alters the regulation of RNAi components and whether such regulation takes place at the transcriptional or translational levels. Finally, given the central role of RNAi in controlling virus infections, it will be important to determine whether RNAi is differentially regulated among different insect strains or during infection with different viruses.

## Long-Term Immune Memory Against Viruses

As discussed above, traces of RNA viral genomes persist in DNA form following clearance of orally acquired viral infections in *D. melanogaster* (79). While similar vDNAs are known to serve as potent sources of siRNAs and are required for the establishment of persistent infections in *D. melanogaster* and *A. albopictus*, it is unknown whether vDNAs play a role in immune priming during responses to previously encountered viruses (34, 79, 80). Future investigations should seek to clarify the role of vDNAs in immune priming, as well as how the routes of prior infections influence susceptibility to subsequent infection via the same or alternate routes.

Another key question is the duration of immune priming following initial pathogen exposure. Does transgenerational immune priming or even heritable immune priming occur? If so, what is the nature of the archived pathogen specificity determinant, and do the mechanisms of its mobilization in subsequent generations differ from those in the generation actually exposed to the pathogen?

While many questions remain regarding the ability of vDNAs to mediate immune memory, it is now clear that vDNAs play a central role in RNAi-based antiviral responses. Recent results indicate that the majority of vDNAs are derived from reverse transcription of defective viral genomes (DVGs), i.e., truncated or rearranged viral genomes produced during viral replication (100). Modulation of DVG production alters the antiviral response in *D. melanogaster*, with higher levels of DVGs being associated with increased vDNA synthesis and an enhanced antiviral response (100). Thus, future studies should examine the dynamics of DVG production during infection, with a particular focus on whether manipulation of DVGs plays a role in long-term immune processes.

## Reprogramming Endogenous Viral Elements as Antivirals

Additional studies are needed to evaluate the role of EVEs in antiviral immune responses, as well as the mechanisms of EVE generation. While genomic integration of vDNAs is a likely scenario for the endogenization of virus sequences, this has yet to be experimentally demonstrated. Furthermore, it is conceivable that different types of viruses (DNA versus RNA) may

become endogenized by one or more alternative mechanisms, and the route of endogenization may influence the functionality of integrated virus sequences. Beyond the mechanisms of EVE generation, the role of EVEs in antiviral responses is still unclear. Given the potential for EVEs to serve as heritable markers of pathogen specificity, it is important to clarify their role during infection with cognate viruses. If it is indeed possible for EVEs to mediate antiviral responses, then their heritable nature provides a potentially valuable means of manipulating insect genomes to modulate susceptibility to virus infection.

## Infectious Clones

While infectious clones for FHV and *Sindbis virus* have proven to be valuable research tools, their extensive host ranges preclude their use as VIGS vectors for pest management. Indeed, the first demonstration in a proof of concept study was for the use of an FHV-based vector in *Drosophila* (122). Infectious clones available for bona fide insect viruses include those for CrPV (53), which has a broad host range, and *Deformed wing virus* of honey bees (60, 107). The lack of insect virus infectious clones results in part from the lack of virus-free cell lines derived from key crop pests that would support replication of candidate viruses of interest. The development of infectious clones for the mosquito-specific flaviviruses *Niénokoué virus* (49) and *Eilat virus* (88) may ultimately allow for their use in combatting mosquito-borne disease (including flaviviruses dengue and Zika) or reducing mosquito populations through antiviral RNAi-mediated targeting.

# CONCLUSION

While significant progress has been made toward increased understanding of the interplay between insect viruses and the host immune response, significant challenges remain to be addressed, not only from the mechanistic point of view (how virus infection is recognized, how the host nutritional status and stress affect the antiviral RNAi-mediated response), but also from a translational perspective for future application of this knowledge in the control of agricultural pests and vectors of diseases, as well as the protection of beneficial insects. There is tremendous potential for powerful biotechnological applications to result from research on insect RNAi pathways.

### SUMMARY POINTS

1. The host siRNA pathway and, possibly, the piRNA pathway are involved in antiviral defense against insect viruses.
2. Both DNA and RNA viruses have evolved suppressors of the siRNA pathway. These suppressors are most important for RNA virus replication, reflecting the primary role of the siRNA pathway in antiviral defense against RNA viruses.
3. As a nucleic acid–based immune system, the siRNA pathway confers long-term immune memory against the same pathogen. This memory may be inherited by the progeny.
4. Virus-derived decoy RNAs may provide an alternative mechanism for evasion of siRNA-based antiviral defense.
5. Virus-derived miRNA can manipulate the host genome to benefit virus replication.
6. Virus-derived DNA sequence, from which transcripts feed into the siRNA pathway, provides insects with immune memory.

7. Virus-derived DNA sequences that are integrated into host genomes, inherited, and retained as endogenous viral elements produce piRNAs that may function in antiviral immunity.
8. The interplay between insect viruses and RNAi has been exploited for practical purposes, including optimization of the baculovirus expression vector system and use of VSRs to detect covert viruses in insect cell lines.
9. Infectious clones of insect viruses have proven invaluable for the study of virus–host molecular interactions and may have future utility for VIGS-based suppression of insect pests and arboviral disease.



## DISCLOSURE STATEMENT

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