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Regulatory role of cellular and viral microRNAs in insect–virus interactions

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The discovery of microRNAs (miRNAs) and their functions has led to a paradigm shift in our understanding of the regulation of gene expression, adding an extra layer of complexity for the mechanisms of gene expression. Both cellular and virus encoded miRNAs play important roles in virus–host interactions that may affect virus replication and the outcome of infection. Recent developments in RNA-seq platforms and bioinformatics tools have accelerated the discovery of miRNAs, their targets, and a myriad of associated research in various species. Here, recent findings and developments in miRNA research pertinent to insect host–virus interactions are reviewed and analyzed.

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Introduction

MicroRNAs (miRNAs) are ~22 nucleotide small non-coding RNAs (sncRNAs) that are produced in most eukaryotes [1], but also by viruses [2] and possibly bacteria [3,4]. These multi-tasking molecules have been shown to regulate transcription and translation of genes involved in almost all cellular pathways. The first miRNA, lin-4, described in *Caenorhabditis elegans* in 1993 was found to regulate the lin-14 gene controlling timing of development [5••]. Since then, thousands of miRNAs have been described and their sequences deposited in the miRNA database, miRBase [6], including many from different insect species. Along with the discovery of more miRNAs, our understanding of their biogenesis is expanding. For instance, in addition to the canonical pathway of miRNA biogenesis (Figure 1), several non-canonical pathways have also been described. These include Drosha-independent, but Dicer-dependent or Dicer-independent, production of miRNAs from introns (known as mirtrons) [7], small nucleolar RNAs [8], transfer RNA [9] and endogenous short interfering RNAs (endo-siRNAs) [10,11]. For detailed miRNA biogenesis pathways, in particular in insects, readers are referred to other recent reviews [12,13,14,15••].

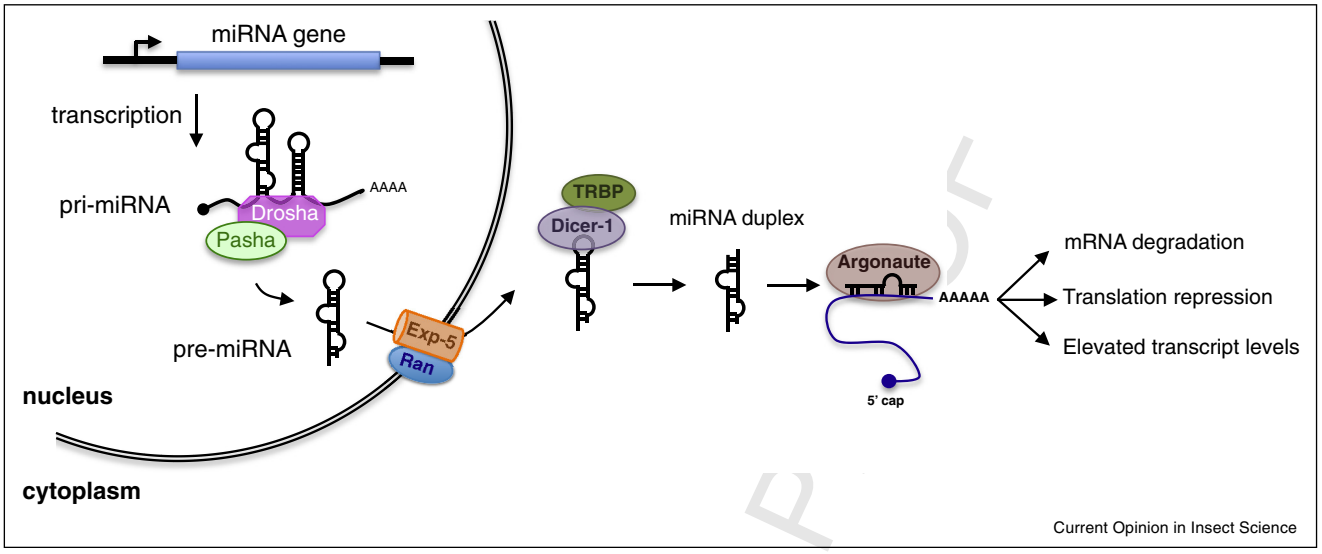
New findings in miRNA research have also provided evidence for non-canonical miRNA–target interactions. For instance, evidence suggests that first, miRNA-target interaction may not always lead to suppression by cleavage of the target mRNA or translational repression, but could also enhance target transcription, by inducing transcription [16•] and translation [17], repressing nonsense-mediated RNA decay [18] or increasing mRNA stability [19]; second, although the majority of target sites of miRNAs might be localized to the 3′UTR of target genes, the 5′UTR and open reading frame (ORF) may also frequently contain non-canonical miRNA binding sites (e.g. [20,21,22,23•]); third, complementarity of the seed region (nucleotides 2–8 from the 5′ end of the miRNA) with target sequences is important in many miRNA-target interactions, but accumulating evidence shows that strong base-paring at the 3′ end or centered pairing may compensate for low complementarity in the seed region (e.g. [21,24]).

Drosophila melanogaster as a model insect with many genetic tools available for its manipulation has been the main subject of miRNA research particularly relating to insect development [12]. In comparison, there is little known about the role of miRNA in insect host-pathogen interactions [25]. In insects, following viral infection the host antiviral responses are activated including the RNA interference (RNAi) pathway, which leads to the production of viral short interfering RNAs (vsiRNAs) that target viral genomes, replication intermediates and transcripts [26,27]. Concurrently, cellular miRNAs, as part of the RNAi response, may target viral genes. Conversely, virus-encoded miRNAs might target cellular or viral genes to facilitate virus replication. Below, the latest developments in miRNA research with respect to insect–virus interactions are reviewed, highlighting issues that require further consideration and analyses.

What is the effect of virus infection on the host miRNA profile?

It is evident from several studies that the host miRNA profile is altered following infection, which may range from small changes to more profound effects depending

Figure 1



Canonical pathway of miRNA biogenesis. A miRNA gene is transcribed by RNA polymerase II/III in the nucleus forming the primary miRNA (pri-miRNA) transcript, which has a 5′ cap and a poly(A) tail similar to mRNAs. Pri-miRNA may contain one or several stem-loop structures. The stem-loop is cleaved near the base by the ribonuclease enzyme Drosha in association with Pasha producing the precursor miRNA (pre-miRNA), which is about 70 nt in length. Pre-miRNA is then transported into the cytoplasm by Exportin-5 (Exp-5) and its co-factor Ran. In the cytoplasm, Dicer-1 together with TRBP removes the hairpin head producing a miRNA duplex. The duplex becomes loaded into one of the argonaute (Ago) proteins (usually Ago1 or Ago2) forming the miRNA-RISC (RNA Induced Silencing Complex). One of the strands (often referred to as miRNA* or passenger strand) may become degraded or alternatively loaded into an Ago protein. The mature miRNA guides the miRNA-RISC complex to target mRNA sequences by sequence complementarity leading to degradation of the miRNA, increased stability or repression of its translation.

on the host and virus combination. Differential expression of host miRNAs has been shown for baculoviruses [28,29,30•], an ascovirus [31], a cytoplasmic polyhedrosis virus [32], West Nile virus (WNV) [33•], chikungunya virus [42] and dengue virus (DENV) [34]. These changes, detected by microarray or deep sequencing analyses, could be either due to host response to viral infection or host manipulation by the virus, including virus-encoded miRNAs (see below). Interestingly, a recent study showed that poxviruses, including the *Amsacta moorei* entomopoxvirus, induce the degradation of the host miRNAs by polyadenylation with a virus-encoded poly(A) polymerase [35•]. This leads to the degradation of the polyadenylated host miRNAs; however, siRNAs are resistant to this mechanism of degradation because they are protected by 2′O-methylation.

While differential abundance of host miRNAs has been documented upon infection in a number of systems, the role of those differentially expressed miRNAs remains to be explored by experimental approaches. In addition, disabling the miRNA biogenesis pathway by loss-of-function mutants (if the technology available) or by silencing key genes in the pathway (such as Drosha or Dicer-1) may allow for elucidation of the extent to which miRNAs contribute to host–pathogen interactions, because differential abundance of host miRNAs upon infection may not necessarily mean that they play a significant role. However, miRNAs produced through non-canonical pathways may not be affected. The Argonaute 1 (Ago1) gene may not be the best choice for silencing to interrupt the pathway because recent evidence shows that miRNAs may also be sorted into Ago2 [7–10]. Relevant to this topic, findings from a recent study provided some insights into the dynamics of miRNA–target interactions, which may have implications for studies investigating differential abundance of miRNAs. Using *Drosophila* S2 cells, it was revealed that although miRNA abundance is overall positively correlated with target repression, changes in the levels of miRNA abundance might not necessarily lead to changes in target levels [36]. For example, miRNAs were identified that even with 2–3-fold changes in abundance still repressed their target to the same level; conversely, miRNAs with similar abundance exhibited differences in their target repression. Consequently, the authors cautioned that miRNA levels *alone* might not be used as a reliable indicator of miRNA function. Further, they demonstrated that miRNA*s that exist at substantially lower levels in comparison with their abundant counterparts from the other arm of the stem-loop also function as repressors of gene expression [36]. In other words, lower abundance of a miRNA does not necessarily result in lower repression of the target gene. For example, the miRNA bantam that is well known for its role in various functions in insects produces two mature miRNAs, bantam-5p being 50-fold

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less abundant than bantam-3p. However, it was shown that bantam-5p is a more active suppressor of the target sequence than bantam-3p [36].

Cellular miRNAs targeting viral or host genes

As indicated above, host miRNAs differentially expressed upon viral infection might potentially target viral genes as part of the host antiviral response. However, viruses may in turn evolve mechanisms to nullify the antiviral effect of the miRNAs or use it to their own advantage. For example, a miRNA (Hz-miR-24) from *Heliothis zea* fat body cells (HzFB) was found to target two subunits of the RNA polymerase genes from *Heliothis virescens* ascovirus (HvAV3e), but the virus suppresses the miRNA abundance by 4-fold during early hours of infection to avoid the impact of the miRNA on transcription of early viral genes that are essential for virus replication [31].

In *Aedes albopictus*, an abundant cellular miRNA, alb-miR-252, is induced 3-fold following DENV-2 infection [37]. The investigators found that inhibition of the miRNA using antagomirs slightly enhanced virus replication (1.5-fold) whereas oversupply of the small RNA (mimic) led to moderately less accumulation of viral genomic RNA (2.5-fold). The gene encoding the viral envelope protein (Protein E) was found to be a target of alb-miR-252. It was suggested that alb-miR-252 could be part of the mosquito's antiviral response.

aae-miR-2940 is a miRNA that based on the current knowledge appears to be mosquito-specific. The 5p arm of the miRNA (aae-miR-2940-5p) was selectively downregulated upon West Nile virus (WNV) infection in mosquito *Ae. albopictus* C6/36 cells [38•]. A target of aae-miR-2940-5p is the *metalloprotease m41 FtsH* gene (MetP), which is positively regulated by the miRNA [39]. Interestingly, MetP was found to enhance WNV replication [38•]. Therefore, reduction of aae-miR-2940-5p abundance after WNV infection leads to lower MetP levels in the cell resulting in reduced virus replication. Given that other cellular miRNAs tested were not affected, the decline in aae-miR-2940 is not due to a global decline in miRNA biogenesis but a selective response. This suggested a miRNA-dependent antiviral response to limit viral replication [38•].

Virus encoded miRNAs and challenges pertinent to RNA virus encoded miRNAs

Virus-encoded miRNAs might target host as well as viral genes (summarized in Figure 2). Those that target host genes may interfere with host miRNA biogenesis, cell proliferation and survival, anti-viral responses, or facilitate virus replication. Viral miRNAs that target virus genes are mainly involved in the regulation of virus replication, which may include switching between lytic and latent viral phases.

DNA viruses

So far, no miRNAs have been reported from cytoplasmic DNA viruses that infect vertebrates or invertebrates. The first insect virus-encoded miRNA was reported from the ascovirus HvAV3e produced from a stem-loop in the ORF coding for the capsid protein. HvAV-miR-1 was shown to target the viral *DNA polymerase I* gene [40••]. From baculoviruses, the *Bombyx mori* nucleopolyhedrovirus encoded BmNPV-miR-3 regulates p6.9 and a number of other late genes [41], and the *Autographa californica* multiple nucleopolyhedrovirus encoded AcMNPV-miR-1 suppresses the ODV-25 gene regulating occlusion-derived virus (ODV) production [42]. All of these examples demonstrate the involvement of virus-encoded miRNAs in autoregulating replication of the viruses mainly during the late phase of infection. This strategy may benefit the viruses by avoiding over-replication within a short period of time, which may compromise host survival, in order to produce more virions over longer period of time to facilitate viral dissemination in the environment.

The only example showing the role of virus-encoded miRNAs in insect virus entry into latency is from *H. zea* nudivirus (HzNV-1) producing two miRNAs, hv-miR-246 and hv-miR-2959, from its non-coding gene (*page1*, persistency-associated gene 1) that downregulate an early gene *hhi1* [43•]. Given that a number of other viruses have been found in insect populations existing in latent infections (e.g. [44]), it will be interesting to explore whether miRNAs could be involved in their latency or reactivation.

As compared with mammalian viruses, there are relatively few examples of virus-encoded miRNAs available that target host genes. From insect DNA viruses, the only example is BmNPV-miR-1, which targets *Ran*, the cofactor of Exportin-5 [30•]. Considering the importance of the export of pre-miRNA from the nucleus to the cytoplasm by Exportin-5 in association with Ran, suppression of Ran by the miRNA leads to a global reduction in mature host miRNAs. However, it is not clear how the virus continues to produce miRNAs, while the biogenesis of host miRNAs is interrupted.

RNA viruses

It was earlier believed that production of miRNAs from RNA viruses is unlikely, mainly due to the fact that the genome or the replicative forms of the viruses could be destroyed via complementary binding of virus-encoded miRNAs, and that most RNA viruses replicate in the cytoplasm where they have no access to Drosha [45••,46]. However, experimental evidence demonstrated that exogenous miRNAs could be produced from cloned pre-miRNAs by recombinant RNA viruses without a negative effect on the virus genome (e.g. [47••]), and access to the nucleus may not be required as viral infection could lead to relocation of Drosha into the cytoplasm

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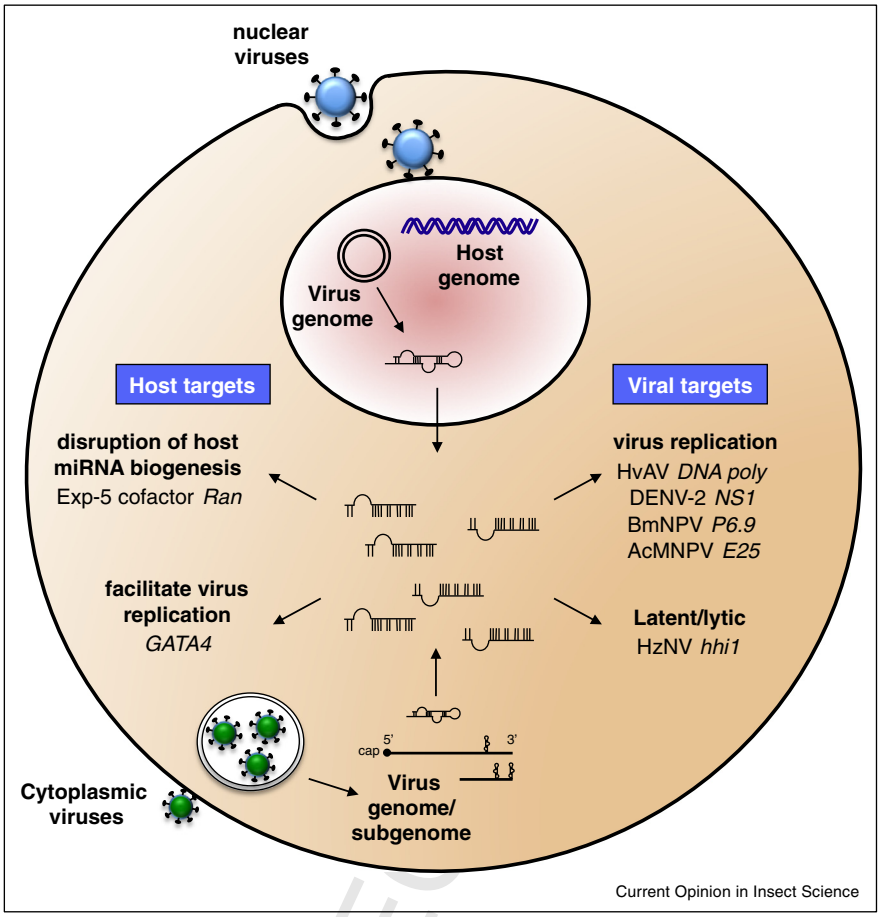
Figure 2



Virus-encoded miRNAs could be produced by nuclear or cytoplasmic viruses through canonical or non-canonical pathways. The mature miRNA that may target the host and/or viral genes. Insect virus-encoded miRNAs that target viral genes generally regulate replication of the virus or the switch between the latent to the active phase by affecting genes that are important for virus replication, whereas those that target host genes may interfere with host miRNA biogenesis or facilitate virus replication. In the figure, examples of target genes (in italic) of insect virus encoded miRNAs and their overall function (in bold) are shown.

[48]. These findings coincided and were followed by a number of publications reporting miRNAs encoded by several RNA viruses. The first RNA virus encoded miRNAs were reported from a retrovirus (HIV-1; reviewed in [49,50]), but those have been challenged [49,51] most notably due to low read numbers of the small RNAs detected in deep sequencing.

From RNA viruses that infect insects, functional miRNA-like viral small RNAs (vsRNAs) have been reported only from flaviviruses, WNV (KUN-miR-1) [52••] and DENV (DENV-vsRNA-5) [53•]. These are produced from stem-loop structures in the 3′UTR of the viral genomes and the subgenomic flavivirus RNA (sfRNA), which mainly consists of the viral 3′UTR. While KUN-miR-1 enhances WNV replication by positively regulating its target, the GATA4 transcription factor, DENV-vsRNA-5 appears to target the non-structural protein 1 (NS1) coding region thereby autoregulating virus replication. However, similar to HIV-encoded miRNAs, low copy numbers in deep sequencings of human and a mosquito cell line (Aag2) has led some to conclude that these viruses do not encode miRNAs and the detected small RNAs are likely the product of another RNAi pathway in the host [54–56]. Deep sequencing indicates these small RNAs are present in low numbers, which could be due to sequencing bias (e.g. [57–60]; also see below). However, several lines of experimental evidence suggest that these viral small RNAs could be functional. For example, first, the precursor stem-loops and mature small RNAs of both KUN-miR-1 and DENV-vsRNA-5 were detectable on northern blots, both with sizes in the range of pre-miRNAs and mature miRNAs. Deep sequencing results are mostly validated by northern blot or stem-loop RT-PCR; second, siRNAs are usually 2′O-methylated blocking polyadenylation, but both KUN-miR-1 and

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DENV-vsRNA-5 were cloned by polyadenylation of the small RNAs in the first step. Notably, several viral-encoded miRNAs have been identified and cloned based on predications and subsequent cloning (e.g. [40••,43•,61••]), without any deep sequencing; third, both small RNAs were detectable in Dicer-2 (Dcr-2)-null C6/36 cells; fourth, inhibiting or overexpressing the small RNAs specifically affected their predicted target and also viral replication; fifth, both small RNAs could be produced from cloned precursor sequences (pre-miRNAs) independent of the virus; sixth, silencing Dcr-2, the enzyme responsible for processing siRNAs, did not affect levels of either KUN-miR-1 or DENV-vsRNA-5; seventh, probes to the other arm of the stem-loop did not detect any small RNAs. miRNAs often appear within a particular period of time and there are some that are highly expressed and others in lower copy numbers. Therefore, while deep sequencing is a powerful technique in providing a starting dataset, experimental approaches provide empirical evidence for data validation, and for ensuing functional analyses of small RNAs. In this regard, in addition to the other reports mentioned above pertinent to biases in deep sequencing data, an elegant comparative study recently showed that several factors involved in sequencing and cloning protocols can deeply influence representation of miRNAs in libraries utilized for high throughput sequencing. For example, mdv1-miR-M7-5p from Marek's disease virus 1 (MDV-1) was 100 times less abundant through the deep sequencing approach compared to small scale sequencing and northern blot analysis [62]. The investigators similarly suggested that various techniques such as northern blot analysis, RT-qPCR and microarray should be used to validate and support deep sequencing data.

Conclusions

Discovery of different types of functional small noncoding RNAs has had a remarkable impact on our understanding of various biological processes and how they are regulated. Cumulative evidence suggests that cellular as well as viral encoded miRNAs play key roles in host–virus interactions to varying extents. These studies have been aided by developments in sequencing technologies allowing deeper analysis of the transcriptome and miRNAome, and bioinformatics tools that are becoming more and more user friendly for biologists. Continued improvements in these areas are essential to allow better and more acceptable analysis of data. For example, reduction in expenses associated with next generation sequencing allows for increased replication. While more investigations present evidence of differential expression of host miRNAs following infection, studies are required to determine the biological significance of these changes with regard to host-virus interactions, target genes, and their roles in regulatory networks. Another suggested area of future study is the comparison of different microbial infections on the same or different hosts, in order to find out if common patterns are observed in regards to the differential expression of miRNAs. Still another area of interest is virus-encoded miRNAs. While the idea that DNA viruses encode miRNAs is readily accepted, production of miRNAs by RNA viruses remains controversial. With regard to this, it might be useful to consider empirical evidence as well as sequencing information when drawing conclusions about the nature of miRNA-like sequences encoded by RNA viruses. Analysis of multiple samples from different stages following infection, in combination with experimental validation, may also help to clarify this issue. While our understanding of the role of miRNAs in insect host-virus interactions is limited, it is important to continue exploring the contributions of miRNAs to these interactions.

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This paper provided the first evidence that cytoplasmic RNA viruses have the potential to produce miRNAs.

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