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Small RNA populations for two unrelated viruses exhibit different biases in strand polarity and proximity to terminal sequences in the insect host *Homalodisca vitripennis*

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ABSTRACT

Next generation sequence analyses were used to assess virus-derived small RNA (vsRNA) profiles for *Homalodisca coagulata virus-1* (HoCV-1), family *Dicistroviridae*, and *Homalodisca vitripennis reovirus* (HoVRV), family *Reoviridae*, from virus-infected *H. vitripennis*, the glassy-winged sharpshooter. The vsRNA reads were mapped against the monopartite genome of HoCV-1 and all 12 genome segments of HoVRV, and 21 nt vsRNAs were most common. However, strikingly contrasting patterns for the HoCV-1 and HoVRV genomic RNAs were observed. The majority of HoCV-1 vsRNAs mapped to the genomic positive-strand RNA and, although minor hotspots were observed, vsRNAs mapped across the entire genomic RNA. In contrast, HoVRV vsRNAs mapped to both positive and negative-sense strands for all genome segments, but different genomic segments showed distinct hotspots. The HoVRV vsRNAs were more common for 5′ and 3′ regions of HoVRV regions of all segments. These data suggest that taxonomically different viruses in the same host offer different targets for RNA-antiviral defense.

Introduction

RNA interference (RNAi) is a natural cellular process for regulating gene expression and providing an innate defense mechanism against invading viruses and transposable elements in diverse host types. Small RNAs are hallmarks of RNAi activity but different types of small RNAs originate by distinct biogenesis pathways. Thus, small RNAs are classified into three major groups: small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi interacting RNAs (piRNAs) (Ding and Lu, 2011). Among these three groups, siRNAs and miRNAs are primarily 20 to 24 nt in length and are processed by double-stranded RNA (dsRNA)-specific Dicer nucleases while piRNAs (23 to 30 nt) are processed in a Dicer-independent manner (Ding and Lu, 2011). Small RNA duplexes from partial or perfect dsRNA precursors are generated by RNase III family enzymes through sequential endonucleolytic cleavage events (Czech and Hannon, 2011). The resulting products are duplex ~20–24-nt small RNAs consisting of two strands. These small RNAs feature 5′ monophosphates and 2-nt overhangs that have hydroxyl groups at the 3′ termini (Ding and Lu, 2011). Independent of their biogenesis pathway, all three groups of small RNAs associate with Argonaute proteins (AGOs) to mediate RNA cleavage or translational repression (Siomi et al., 2011).

RNAi activity in plants, fungi and nematodes largely depends on amplification of siRNAs by respective cellular RNA-dependent RNA polymerases (RdRPs), which are largely absent in fruit flies, mosquitoes and other insects. However, virus infection of *Rift Valley fever virus* (RVFV) infected mosquito cells resulted in the production of virus-derived small RNAs (vsRNAs) that act as siRNAs (Leger et al., 2013). RNAi activities play significant roles in protecting many organisms, including insects, against infecting viruses (Belles, 2010; Huvenne and Smagghe, 2010). Consequently, this anti-viral RNAi activity may generate a large population of vsRNAs within the virus-infected host. Recent studies using virus-infected cell lines and whole insects have shown that vsRNAs corresponding to both siRNAs and piRNAs may be common (Hess et al., 2011; Leger et al., 2013; Morazzani et al., 2012).

Recent advancements in next generation sequencing (NGS) technologies, such as RNAseq, have led to new opportunities to analyze large populations (tens of millions) of small RNAs (Schuster, 2008; Varshney and May, 2012), including those derived from viral genomes. In virus-infected hosts this has facilitated virus discovery and provided new means to validate and/or characterize virus infections (Gausson and Saleh, 2011; Wu et al., 2010). One recent small RNA study of soybean aphid (*Aphis glycines*) (Liu et al., 2011) resulted in the identification of a novel

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ssRNA positive-strand virus (*A. glycines virus*, AGV), and two other known aphid viruses (*Aphid lethal paralysis virus*, ALPV; *Rhopalosiphum padi virus,* RhPV). Furthermore, NGS of small RNAs from *Dengue virus*-infected mosquitoes showed differences in quality and quantity of small RNAs over time (Hess et al., 2011; Scott et al., 2010).

The glassy-winged sharpshooter, *Homalodisca vitripennis* (Hemiptera: Cicadellidae), is a xylem-feeding leafhopper and an important pest on a wide range of plants including *Citrus* spp., grapes (*Vitis vinifera*) and almonds (*Prunus dulcis*) (Blua and Morgan, 2003; Redak et al., 2004). *H. vitripennis* also serves as a vector for *Xylella fastidiosa,* the causal agent of Pierce's disease of grapevines and citrus variegated chlorosis disease (Backus et al., 2012). Natural populations of *H. vitripennis* harbor two unrelated viruses: *Homalodisca coagulata virus-1* (HoCV-1) of the family *Dicistroviridae* (Hunnicutt et al., 2006) and *H. vitripennis reovirus* (HoVRV) of the family *Reoviridae* (Stenger et al., 2010; Stenger et al., 2009).

HoCV-1 has a monopartite, positive-sense ssRNA genome (9321 nt, exclusive of the polyadenylated 3′-terminus) encoding two large open reading frames located between nt 420–5807 (ORF1) and 5990–8740 (ORF2) (Hunnicutt et al., 2006). In contrast, the genome of HoVRV is composed of 12 dsRNA segments (Stenger et al., 2009). HoVRV genomic dsRNAs, similar to those of other reoviruses, have conserved segment termini at the 5′-ends (GGCG or GGCA) and 3′-ends (UGAU or CGAU) of the positive sense strand; adjacent imperfect inverted repeats have the potential to base pair (Stenger et al., 2009). Other members of the genus *Phytoreovirus* have host ranges including both plants and insects, but HoVRV is only known to infect *H. vitripennis* (Stenger et al., 2009).

Despite accumulating to high titers, HoCV-1 and HoVRV do not cause obvious disease symptoms in *H. vitripennis*. Indeed, some of our *H. vitripennis* colonies are infected by both viruses, yet such colonies remain robust. These colonies provided an opportunity to investigate patterns of vsRNAs derived from two taxonomically distinct viruses in a single insect host. Here, we used high-throughput small RNA sequencing to map and compare populations of RNAi-induced vsRNAs for HoCV-1 and HoVRV in *H. vitripennis*. Interestingly, vsRNA populations derived from HoCV-1 and HoVRV were distinct from one another with respect to bias in strand polarity and proximity to terminal sequences, suggesting that these two taxonomically unrelated viruses offer different RNAi targets during infection of *H. vitripennis*.

Results

Analysis of small RNAs from HoCV-1 and HoVRV-infected H. vitripennis

Sequencing of adult *H. vitripennis* small RNA libraries yielded 22,151,482 reads (Fig. 1a). Small RNA sequencing reads (43% of the total, ~9.5 million) were mapped to an artificial build of the *H. vitripennis* transcriptome (Nandety and Falk, unpublished). Sequences that matched to redundant rRNA and tRNA sequences were eliminated in subsequent steps to generate the unique datasets. Approximately 18% of the total small RNA reads (4,186,078) were unique to the *H. vitripennis* genome (Fig. 1a). Approximately 57% of the small RNA reads (12,620,344) that did not match the *H. vitripennis* transcriptome, upon further analysis, revealed information on biologically significant associated microbes, including viruses and bacteria.

The majority of small RNA sequences from the *H. vitripennis* sequencing libraries were 19–27 nt in length, with the predominant size class being 21 nt (Fig. 1b). These classes of small RNAs are typical for Dicer (Dcr-2) derived products (Ding and Lu, 2011). The length distributions of the small RNA product classes was seen for *Bombyx mori* (silk worm), *Nilaparvata lugens* (brown plant hopper) (Wei et al., 2009) and for *Dengue virus* (DENV-2) infected mosquito cell lines (Hess et al., 2011). The predominance of 21 nt vsRNAs in *H. vitripennis* and *N. lugens* suggests that small RNA biogenesis pathways in these two leafhoppers are likely conserved.

Identification of small RNAs from H. vitripennis viruses

Adult *H. vitripennis* were used as a source of siRNAs to identify viruses infecting *H. vitripennis*. High quality small RNA contigs ($>$ 100 nt) were used as queries in BLAST searches. The resulting BLAST identities were tabulated using perl scripts that identified major identity viral "hits" to HoCV-1 and HoVRV. Two contigs that matched *Taastrup virus* and a single contig match to *Taura syndrome virus* (TSV) also were identified but due to low abundance, these sequences were not further analyzed. HoCV-1 and HoVRV were widely represented in the BLAST results; vsRNAs completely covered the respective virus genomic RNAs. Each of the contigs that had a proper identity match to the known HoCV-1 and HoVRV genomes is represented as an identifier. Presence of HoCV-1 and HoVRV in the source insects also was validated via RT-PCR as described (Hunnicutt et al., 2006; Stenger et al., 2009).

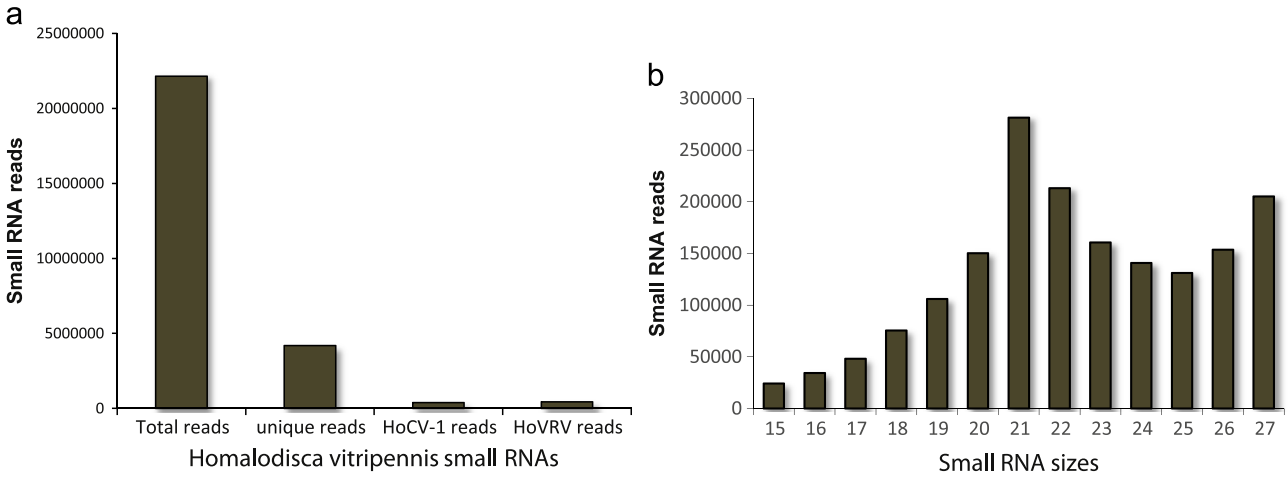

Fig. 1. Small RNA sequencing summary from *Homalodisca vitripennis* adult insects: (a) Graphical summary of total and unique small RNA reads generated from *H. vitripennis* libraries along with the number of small RNA reads that are HoCV-1 and HoVRV specific. (b) Graphical summary of small RNA size classes of the *H. vitripennis* small RNA reads.