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Plant Viruses Transmitted in Two Different Modes Produce Differing Effects on Small RNA-Mediated Processes in Their Aphid Vector

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Accepted for publication 27 November 2018.

ABSTRACT

Transmission of plant viruses by aphids involves multitrophic interactions among host plants, aphid vectors, and plant viruses. Here, we used small RNA (sRNA) sequencing to visualize the sRNA response of *Myzus persicae* to two plant viruses that *M. persicae* transmits in different modes: the nonpersistent *Potato virus Y* (PVY) versus the persistent *Potato leafroll virus* (PLRV). Aphids exposed to PLRV produced significantly less 22 mers aligned to the aphid genome, and an abundance of 26 to 27 mers, many of which were predicted to be piRNA. Additionally, expression of *Buchnera aphidicola* tRNA-derived sRNAs was influenced by PLRV and, to a lesser extent, PVY,

suggesting that plant viruses alter the aphid-endosymbiont relationship. Finally, aphids exposed to PLRV-infected plants generated an abundance of unusually long sRNAs and a reduced number of 22 mers against an aphid virus, *Myzus persicae* densovirus (MpDNV) and had higher MpDNV titer. Expression of the PLRV silencing suppressor P0 in plants recapitulated the increase in MpDNV titer in the absence of PLRV infection. Our results show that plant viruses transmitted in two different modes cause distinct effects on their vector with regards to post-transcriptional gene regulation, symbiosis with *Buchnera*, and the antiviral immune response of aphids to an aphid-infecting densovirus.

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Data Availability: The datasets supporting the conclusions of this article have been made available to the research community in the NCBI Short Read Archive (SRA) and can be found under BioProject PRJN514359.

P. V. Pinheiro and J. R. Wilson contributed equally to this work.

Funding: Funding for the present research was provided by National Science Foundation grant 1354309 (to M. Heck) and Graduate Research Fellowship DGE-1650441 (to J. R. Wilson) and by an Empresa Brasileira de Pesquisa Agropecuária Graduate Student Fellowship (to P. V. Pinheiro). DNA sequence data for *Myzus persicae* were downloaded from AphidBase, <http://bipaa.genouest.org/is/aphidbase/>. Funding for *Myzus persicae* clone G006 genomic sequencing was provided by USDA-NIFA award 2010-65105-20558. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

*The e-Xtra logo stands for “electronic extra” and indicates that six supplementary figures and three supplementary tables are published online.

The author(s) declare no conflict of interest.

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Aphids are the most important and widespread vectors of plant viruses (Nault 1997; Ng and Falk 2006). Together, aphids and the plant viruses they transmit cause significant crop yield losses around the world. Transmission of plant viruses by aphids involves coevolved, multitrophic interactions among the plant host, the aphid vector, and the plant virus. In the molecular tug-of-war of plant virus infection and transmission, plant hosts activate their immune defenses against aphids and plant viruses, and the latter two use a myriad of strategies to overcome the host plant defenses. For instance, plant viruses encode silencing suppressor proteins to impair the plant antiviral immune system (Ding and Voinnet 2007; Li and Ding 2006; Mlotshwa et al. 2008).

Aphid-borne plant viruses are transmitted by their aphid vectors via distinct modes of transmission, such as nonpersistent, stylet-borne or persistent circulative (Nault 1997). For instance, the green peach aphid, *Myzus persicae*, can transmit over 100 different plant viruses, including the persistent, circulative *Potato leafroll virus* (PLRV) and the nonpersistent *Potato virus Y* (PVY), which both infect plants in the family Solanaceae (Kennedy et al. 1962).

Nonpersistent viruses do not circulate through the insect vector tissues when transmitted. Upon ingestion, nonpersistent viruses stay bound to the aphid stylet for a short period where they can be transmitted to a new host plant upon probing. On the other hand, persistently transmitted viruses, such as the plant viruses in the family *Luteoviridae*, referred to as luteovirids hereafter in this paper, circulate through the insect vector tissues to be transmitted to a new host plant. Luteovirids are transmitted exclusively by aphids. Once a luteovirid is acquired, aphids remain viruliferous for their entire lives (reviewed in Gray et al. 2014).

The vector manipulation hypothesis has been proposed to explain the evolution of strategies that optimize the plant-to-plant spread of pathogens by vectors through influencing the plant host selection and feeding behavior of the insect vector (Ingwell et al. 2012; Mauck et al. 2012). It is known that both persistent and non-persistent viruses alter the host plant to enhance transmission by insect vectors, but they use different strategies consistent with their mode of transmission. For instance, aphids are attracted to plants infected with persistent and nonpersistent viruses, but upon feeding, they perceive plants infected with a nonpersistent virus as a poor source of food and only transiently probe before finding another host plant (Alvarez et al. 2007; Castle et al. 1998; Mauck et al. 2010, 2012). However, this brief interaction is sufficient for virions to bind the stylet and be carried to the next host. Other effects of vector manipulation by circulative viruses have been extensively reported, such as an increase in longevity and reproduction rates of the vector (Castle and Berger 1993; MacKinnon 1961; Pickett et al. 1992). It has been suggested that insect vectors and the viruses they transmit collaborate in fighting or avoiding host plant defenses (Hodge and Powell 2008, 2010; Jiu et al. 2007; Kersch-Becker and Thaler 2014).

In addition to interactions with plant viruses, aphids, like the majority of other hemipteran insect vectors, also harbor close associations with both obligate and commensal microbes (Baumann 2005; Guyomar et al. 2018) as well as insect pathogens, such as insect viruses (Feng et al. 2017). For aphids and *M. persicae* in particular, these associations include an obligate bacterial endosymbiont, *Buchnera aphidicola* (phylum gamma-Proteobacteria) (Baumann et al. 1995). Aphids depend on *Buchnera* to provide the essential amino acids that aphids cannot synthesize or obtain from their diet of phloem sap (Hansen and Moran 2011). Using proteomics, amino acid transporters are found in high abundance in the bacteriocytes, specialized insect cells harboring the symbiont (Poliakov et al. 2011). Different genotypes of *Buchnera* have been found in aphid clones that differ in their ability to transmit poleroviruses in the plant virus family *Luteoviridae* (Cilia et al. 2011). It is unlikely that *Buchnera* plays a direct role in plant virus transmission (Bouvaine et al. 2011), but it is unknown how plant infection with aphid-transmitted viruses influences the aphid-*Buchnera* symbiosis. Aphid-infecting viruses have also been identified and characterized (Gildow and D'arcy 1990; Ryabov 2007; Ryabov et al. 2009; Teixeira et al. 2016; van Munster et al. 2002, 2003b; Williamson et al. 1988; van der Wilk et al. 1997), including viruses which have integrated into the aphid genome (Clavijo et al. 2016). In contrast to the luteovirids, which do not replicate in their insect vectors (Day 1955; Eskandari 1979; Harrison 1958; Weidemann 2009), insect viruses replicate in insect cells and produce effects on aphid physiology that alter phenotypes important to the transmission of plant viruses, such as increasing wing production (Ryabov et al. 2009) or decreasing fecundity (Moon et al. 1998; van Munster et al. 2003b). One aphid-infecting virus is *Myzus persicae densovirus* (MpDNV), a single-stranded DNA virus in the family *Parvoviridae*

(van Munster et al. 2003b) with a genome of approximately 5.7 kb (van Munster et al. 2003a). Only minor effects of MpDNV on aphid reproduction and development have been reported (van Munster et al. 2003b). MpDNV is horizontally transmitted via saliva and honeydew, and can also be transmitted transovarially (van Munster et al. 2003b). Interactions with insect-infecting viruses may complicate the interaction of aphids and aphid-borne plant viruses, as both insect and plant viruses require aphids for transmission, but in fundamentally distinct ways. The role insect-infecting viruses play in insect-vectored plant virus transmission remains hitherto unknown, as well as the molecular mechanisms involved in these manipulations.

Based on the well-studied manipulation of insect vector feeding behavior by plant viruses, we hypothesized that plant viruses induce changes in biochemical pathways in their insect vectors and sought to compare these effects on *M. persicae* using small RNA sequencing (sRNA-seq) after aphids acquire plant viruses transmitted in two different modes: nonpersistent (PVY) versus persistent (PLRV). sRNA-seq is a powerful tool that can capture the RNAi pathways involved in host post-transcriptional gene regulation as well as antiviral immunity, which is largely mediated by sRNA in plants and invertebrates, such as aphids (Aliyari et al. 2008). The pathways that regulate RNAi are expanded in aphids (Jaubert-Possamai et al. 2010) and also functional (Jaubert-Possamai et al. 2007; Mutti et al. 2006; Pitino et al. 2011). Viruses replicating in host tissues would cause the host to produce virus-derived small interfering RNA (viRNA), which can be detected via sRNA-seq. We anticipated the aphids will launch an sRNA-mediated antiviral response against MpDNV, an animal virus infecting aphids in our colony, but not against PVY or PLRV, two plant viruses which do not replicate in the aphid vector. Our results show that feeding on plants infected with PLRV and PVY cause differing effects on posttranscriptional gene regulation, symbiosis with *Buchnera*, and the antiviral immune response of aphids to MpDNV, an aphid-infecting virus.

MATERIALS AND METHODS

Aphids and viruses. sRNA-seq was performed on *M. persicae* exposed to plants infected with viruses that varied in their transmission modes (PLRV and PVY). Both plant and aphid samples were collected for sRNA seq. For all experimental conditions, aphids were placed on their source plant tissue or control treatments for 3 days, transferred to turnip for gut clearing, and then collected for sRNA seq (Supplementary Fig. S1). Gut clearing was used for all treatments because a previous experiment performed with no gut clearing prior to sequencing detected abundant plant-derived PLRV reads in aphid samples (H. J. Ju and S. Gray, *unpublished data*), presumably derived from sRNA ingested from the plant sap during feeding.

For these experiments, parthenogenic colonies of the green peach aphid *Myzus persicae* Sultz were maintained on caged *Physalis floridana* at 20°C with an 18-h photoperiod. This *M. persicae* clone was originally collected from New York and maintained in the lab clonally for over 15 years. Aphids were allowed a 3-day virus acquisition access period (AAP) on the following treatments prior to collecting aphids for sRNA-seq: (i) PLRV-infected potato plants, cultivar Red Maria; (ii) purified, 50 µg/µl infectious purified PLRV in 30% sucrose; (iii) PVY-infected potato plants, cultivar Goldrush; (iv) mock-inoculated potato plants, cultivar Red Maria; and (v) 30% sucrose solution. After the AAP, aphids were transferred to turnip plants, cultivar Purple Top White Globe, (a nonhost for PLRV and PVY) for 3 days to clear all insect tissues of plant viRNAs mapping

to PLRV and PVY produced in planta. Aphids were harvested from turnip plants and flash-frozen for sRNA isolation. For each treatment, three to four biological replicates were harvested, each containing a pool of approximately 700 aphids (50 to 80 mg). To ensure that aphids acquired PLRV from the infected plants and the virus-laden diets, a subset of aphids were tested by RT-PCR, using primers that amplify a 660-bp fragment of PLRV, which included the coat protein: (5'-CTAAAGATTCCTCCACGTGCG-3') and (5'-GGAGTGGGTGTTGGTTGTGGC-3').

Tissue was also collected from turnip plants used to gut clear aphids exposed to PLRV (PLRV-infected potato and purified PLRV treatments). Potato and turnip plant samples were collected from aphid-inoculated leaves 3 days postinoculation (DPI) and from systemically PLRV-infected potato leaves 3 weeks post-inoculation (WPI). Turnip samples were only collected 3 days after PLRV inoculation by aphids because turnip does not become locally or systemically infected with PLRV, so sampling at later time points for systemic infection was not necessary.

Three-week-old hairy night shade (HNS, *Solanum sarrachoides*) plants were inoculated with a cDNA clone of PLRV wild type (Franco-Lara et al. 1999) to serve as source of virus for inoculation of potato plants by aphid feeding. Tobacco (*Nicotiana tabacum*) plants infected with PVY strain O (Karasev et al. 2011) were used as inoculum for aphid transmission of PVY to the potato plants used in the experiments. HNS plants were used as a source of virus because they are more easily inoculated with the cDNA clone in *Agrobacterium tumefaciens* (DeBlasio et al. 2015).

sRNA isolation, library construction, and sRNA sequencing. sRNAs were isolated from whole aphids and plant leaves using the mirPremier microRNA isolation kit (Sigma-Aldrich). RNA integrity was confirmed using gel electrophoresis. sRNA libraries were constructed from 50 to 100 ng of sRNA, as described (Chen et al. 2012), with some modifications. A commercial sRNA 3' linker was used for adapter ligation (5'rApp-CTGTAGGCACCATCAAT-Amine 3') (New England BioLabs). The reverse transcription primer 5'Amine-GACGTGTGCTCTCCGATCTATTGATGGTGCCTACA*G 3' was used to hybridize to the excess 3' adapter and convert the single stranded DNA adapter into a double-stranded DNA molecule. Three to four individual sRNA libraries were prepared for each aphid and plant treatment from purified sRNA using unique barcoded-adapters. Individual libraries were separated by gel electrophoresis and 160 to 180 bp sized libraries were selected, as the adapter is 141 bp. Libraries were pooled and quality checked with a bioanalyzer. Libraries were then pooled in four lanes and sequenced on an Illumina HiSeq2500 instrument at the Cornell Biotechnology Resource Center, operating in "High Output Mode" with single-end 50-bp read length.

sRNA data analysis. sRNA deep sequencing data were processed to remove sequencing adapters, low quality reads, and short reads (<15 nt) using the sRNA clean script provided by VirusDetect (Zheng et al. 2017). The remaining reads were aligned to a ribosomal RNA database (Quast et al. 2013) using Bowtie (Langmead et al. 2009) and the mapped rRNA reads were removed. The cleaned sRNAs were mapped to the reference genomes using Bowtie (Langmead et al. 2009) allowing up to 1 mismatch to account for differences between the reference sequences and our aphid clone and virus strains. Reference genomes included: *Potato leafroll virus* (NC_001747 and KC456053), *Buchnera aphidicola* F009 strain from *Myzus persicae* (CP002703), *Myzus persicae* densovirus (AY148187), *Potato virus Y* (EF026074), and *Myzus persicae* G006 (draft genome available at <http://bipaa.genouest.org/is/aphidbase/>). The mapping depth at each position of the MpDNV genome was generated using SAMtools (Li et al. 2009). The cleaned reads were also aligned to the mature tRNAs of

B. aphidicola using Bowtie with 0 mismatches, and the resulting alignments were visualized using Tablet (Milne et al. 2013).

To look for piRNA, sRNA reads of 26 to 27 nt from aphids exposed to PLRV were aligned to the *M. persicae* G006 draft genome using Bowtie allowing no mismatch. Mapped reads of 26 and 27 nt were analyzed using the piRNN deep learning algorithm (Wang et al. 2018) using the *Drosophila melanogaster* model, since this is the best available model for insects. Sites of integrated MpDNV were obtained by aligning the *M. persicae* draft genome G006 to the genome of MpDNV (AY148187) using BLAST (Altschul et al. 1990), with an e-value cut-off of 10^{-5} .

To observe differences in the size distribution of reads mapping to the *M. persicae* or MpDNV genome, the percentage of reads of each size aligning to the reference genome was averaged across biological replicates. Error bars represent one standard error. We compared the distributions of sRNA sizes from the different treatments using methodology based on Kramer (Kramer 2014), modified for multinomial distributions, which allowed us to include multinomial sampling error variance, replicate-to-replicate variance, and treatment-to-treatment variance. The within-treatment variance was created by simulating each replicate within a treatment (e.g., treatment A) from a multinomial distribution using the proportion of sRNA sizes and counts from that replicate and building the null distribution from means (based on four replicates) of the log of sum-of-square differences, with each difference based on two simulated reps. This was done 1,000 times using all replicates of treatment A, providing the null distribution of within-treatment mean differences. The mean of the log of sum-of-square differences between simulated data from all replicates of two different treatments (e.g., treatment B versus treatment A) was then compared with the null distribution of means built from only treatment A simulated data. If this mean lay outside the upper 95th percentile of the within treatment A distribution, the distributions of sRNA sizes from treatment B was declared to differ significantly from that of treatment A. The *P* values were also calculated using treatment B to generate the null distribution and in all cases gave similar *P* values to those from a treatment A null distribution.

To compare the abundance of reads across treatments, a one-way fixed effects analysis of variance (ANOVA) was performed. Tukey's honest significant difference (HSD) test was used to perform pairwise comparisons if the ANOVA test was significant. The assumption of normality was checked using the Shapiro-Wilk test and a normal probability plot. The assumption of homoscedasticity was checked using the Bartlett test. All statistical tests were performed in R (R Core Team 2017).

Quantifying MpDNV titer in aphids allowed an AAP on PLRV-infected or uninfected plants. To test whether PLRV infection in plants impacted MpDNV titer in aphids, we used droplet digital PCR (ddPCR) to measure MpDNV titer in aphids. Aphids were given a 3 day AAP on PLRV-infected or uninfected HNS (*S. sarrachoides*) plants. DNA was extracted from single aphids (20 replicates/treatment), by homogenizing the whole aphid using a micro pestle in 30 μ l of extraction buffer (0.01 M Tris, pH 8.0, 0.001 M EDTA, and 0.025 M NaCl) containing 0.2 μ g/ μ l Proteinase K. The insect homogenate was then incubated at 37°C for 30 min (Proteinase K digestion) followed by 95°C for 2 min (Proteinase K heat inactivation). After Proteinase K treatment, the samples were centrifuged for 7 min at 16,100 $\times$ g. The supernatant was then transferred into a new tube and stored at -20°C until further use. A ddPCR assay was developed for MpDNV using the QX100 droplet digital PCR system (Bio-Rad). The ddPCR reaction for MpDNV consisted of 10 μ l of 2 $\times$ ddPCR Evagreen SuperMix (Bio-Rad), 1 μ l of each 10 μ M MpDNV primer (5'-TGACAATGGGTATATTCATTGACCT-3' and 5'-ATCGTGCGTCAAAAGAAACCCT-3'), 7 μ l of dH₂O and 2 μ l of DNA diluted at

1:800 in a final volume of a 20 µl reaction. A cartridge holder containing 20 µl of the ddPCR reaction and 70 µl of droplet generator oil for Evagreen (Bio-Rad) was placed into the QX100 droplet generator (Bio-Rad) where 40 µl droplets were generated. Droplets were then transferred to a 96-well plate (Eppendorf) and the plate was sealed with an easy pierce foil seal (Bio-Rad). PCR amplification was carried out on the Applied Biosystems 2720 Thermocycler. The thermocycling conditions started at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min, 1 cycle at 4°C for 5 min, 1 cycle at 90°C for 5 min and ending at 12°C. Following amplification, the plate was inserted into the droplet reader cassette and loaded into the droplet reader (Bio-Rad). The droplets were automatically read at a rate of eight wells per 15 min. The ddPCR droplet data were analyzed using the QuantaSoft analysis software (Bio-Rad), which presents the target results as copies per microliter of PCR mixture. The number of copies of MpDNV per microliter was compared between treatments using the Kruskal-Wallis nonparametric test, since the Shapiro-Wilk test for normality was significant ($P < 0.0001$).

MpDNV titer in aphids allowed an AAP on the PLRV silencing suppressor protein, P0. We investigated if the PLRV silencing suppressor P0 was responsible for the increase in MpDNV titer in PLRV-viruliferous aphids using *Nicotiana benthamiana*, which is a host of both PLRV and *M. persicae* and commonly used for transient protein expression in the leaves. To test this hypothesis, aphids were fed upon *N. benthamiana* tissue transiently expressing PLRV, P0, or GFP driven by a 35S promoter or noninfiltrated (healthy) plants. Transient expression was achieved using plant inoculation by *Agrobacterium tumefaciens* as described (DeBlasio et al. 2015). Synchronized fourth instar aphids were placed upon infiltrated leaves 1 DPI, caged on three leaves/plant, five to six plants/treatment. One cage of aphids per plant was collected in pools of four to eight aphids, resulting in 10 to 12 biological replicates per treatment per time point. Insects were homogenized by cryogenic grinding in liquid nitrogen for 6 min at 25 Hz with a Mixer Mill MM 400 (Retsch). DNA was extracted using 150 µl of extraction buffer with Proteinase K treatment, as described above. DNA samples were

normalized to 5 ng/µl and then diluted 1:8. MpDNV titer was quantified using the ddPCR assay described above. The number of copies of MpDNV per microliter was compared among treatments using a one-way fixed effects ANOVA for the first time point (1 day, 2 DPI), and via the Kruskal-Wallis nonparametric test for the second time point (3 days, 4 DPI), since the Shapiro-Wilk test for normal distribution was significant ($P < 0.05$). Titers are presented as copies of MpDNV found in 1 ng/µl of total aphid DNA.

To assess silencing suppressor activity in the plant, as previously described (Shen et al. 2010), *N. benthamiana* plants were coinfiltrated with *A. tumefaciens* cultures expressing GFP, double-stranded GFP, and P0 or WT PLRV. Double-stranded GFP will silence GFP expression and result in no GFP fluorescence, unless silencing suppression occurs. GFP expression and therefore silencing suppressor activity was accessed using a long wavelength UV lamp 4 DPI. The P19 silencing suppressor protein from *Tomato bushy stunt virus* was included as a positive control, and no silencing suppressor for a negative control. Transient expression of all proteins was driven by the 35S promoter.

MpDNV titer in winged and nonwinged aphids. To test whether there was a correlation between densovirus and winged morphs, as has been observed in other aphid species (Ryabov et al. 2009), we quantified MpDNV in winged and nonwinged aphids. Alates and apterous aphids were collected at the same time from the same colony on uninfected *Physalis floridiana* (pools of five aphids, 16 replicates per treatment). DNA was extracted using cryogenic lysis of aphid tissue and extraction buffer as described for aphid feeding upon P0. DNA samples were normalized to 5 ng/µl and then diluted anywhere from 1:8 to 1:200, depending on MpDNV titer in the sample as a result of the incredible range of MpDNV titer found in winged aphid samples. Therefore, data are presented as copies of MpDNV found in 1 ng/µl of total aphid DNA. Average MpDNV titer was compared using the Mann-Whitney nonparametric test ($P = 9.57 \times 10^{-5}$) as data were found to be not normally distributed via the Shapiro-Wilk test ($P = 1.70 \times 10^{-6}$) and heteroscedastic via the Levene test ($P = 0.014$).

TABLE 1
Number of reads obtained by Illumina deep sequencing of small RNAs generated in aphid and plant samples

Tissue	Treatment	Average number of cleaned reads ^a	PLRV ^b	PVY ^c	MpDNV ^d	<i>Buchnera aphidicola</i>	% <i>Buchnera</i> tRNAs ^e
Aphids	PLRV-infected potato ^f	5,673,826	64	79	2,979	47,1394	10.8%
	Purified PLRV in 30% sucrose ^c	5,966,972	48	108	2,845	723,696	13.1%
	Mock-inoculated potato ^f	1,898,702	–	51	2,196	633,184	4.1%
	PVY-infected potato ^f	4,909,636	–	203	6,830	1,594,164	7.3%
	30% sucrose	3,270,474	–	48	13,340	1,182,707	12.5%
	30% sucrose ^f	1,735,400	–	14	6,457	447,774	14.8%
Potato leaves	PLRV-infected, 3 WPI ^g	1,885,542	21,918	13	79	9,187	2.9%
	PLRV-infected, 3 DPI ^h	1,317,272	3,419	3	–	2,203	8.4%
Turnip leaves	Turnips fed to aphids after feeding on PLRV-infected potato	1,158,558	108	33	37	12,988	7.2%
	Turnips fed to aphids after feeding on purified PLRV	1,873,005	169	42	48	9,283	4.2%

^a The average number of cleaned reads reflects the average number of reads per replicate after removal of adapter and rRNA sequences.

^b *Potato leafroll virus* (PLRV).

^c *Potato virus Y* (PVY).

^d *Myzus persicae* densovirus (MpDNV).

^e The percent of reads aligned to *B. aphidicola* that aligned to *B. aphidicola* tRNAs.

^f After the initial treatment, all of these aphids were also moved to turnip for 3 days.

^g WPI, weeks postinoculation.

^h DPI, days postinoculation.

Localization of MpDNV in PLRV-viruliferous aphids by fluorescence in situ hybridization (FISH). FISH was performed as previously described (Kliot et al. 2014). Briefly, specimens were fixed in Carnoy's fixative (chloroform-ethanol-glacial acetic acid, 6:3:1, vol/vol) for 5 min following gut dissection in 1× PBS and hybridized overnight in hybridization buffer (20 mM Tris-HCl, pH 8.0, 0.9 M NaCl, 0.01% [wt/vol] sodium dodecyl sulfate, 30% [vol/vol] formamide) containing 10 pmol fluorescent probe per microliter. For specific targeting of PLRV and MpDNV, PLRV (5'-TTTCCATTTCCCTTCCACAG-3') (Ghanim et al. 2009) and DenR2 (5'-ATCGTGCCTCAAAAGAAACCCT-3') DNA probes were used respectively. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI; 0.1 mg/ml). The stained guts were mounted in hybridization buffer and viewed under a Leica TCS-SP5 (Leica Microsystems Exton) confocal microscope. At least 10 guts were viewed for each treatment under the microscope to confirm reproducibility. Specificity of detection was confirmed using no-probe and uninfected controls.

RESULTS AND DISCUSSION

sRNA sequencing detected an sRNA-mediated antiviral immune response to PLRV in potato, a host of PLRV, but not in turnip, a nonhost, or *M. persicae*, the aphid vector. Aphids were allowed an AAP on PVY-infected potato plants, PLRV-infected potato plants, purified PLRV virions delivered via a sucrose diet, and a purely sucrose diet. After a 3-day AAP, aphids were transferred to turnip plants for 3 days for gut clearing. Potato plant and turnip plant samples were collected from aphid-inoculated leaves 3 DPI and from systemically infected potato leaves 3 WPI. Three to four individual sRNA libraries were prepared for each aphid and plant treatment from purified sRNAs using unique barcoded-adapters. Each aphid library generated between 3 and 7 million

reads, while the plant libraries generated between 1 and 2 million reads (Table 1). Aphid and plant libraries generated reads varying from 15 to 40 nt in length, which we refer to in this manuscript as sRNA reads.

viRNA reads that aligned to the PLRV genome were present in PLRV-infected potato at both sampling times (Table 1). The number of viRNA reads that aligned to PLRV in potato at 3 DPI was lower than at 3 WPI, but still readily detectable, which indicates that 3 days is sufficient time to detect PLRV viRNA in plant tissue. The majority of those reads were 21 to 22 nt (Supplementary Fig. S2), which is in the range of the viRNA size reported for plants, including potato (Hwang et al. 2013; Kutnjak et al. 2015; Li et al. 2012). In contrast, the turnip plants used for gut clearing of aphids did not produce a significant number of PLRV-derived sRNA (Table 1), which is consistent with the fact that turnip is a nonhost of PLRV. Therefore, virus replication does not occur in turnip.

No sRNA reads that aligned to the PLRV genome were detected in aphids allowed an AAP on PVY-infected plants and all control treatments (sucrose diet and mock-inoculated potato). Minimal sRNA reads mapping to PLRV were generated in aphids which acquired PLRV from PLRV-infected plants or purified PLRV diet treatments (Table 1). These reads were manually inspected and are considered false positives, as they could also be aligned to other, non-PLRV sequences ranging from aphids, humans, and plants. Similarly, the minimal sRNA reads mapping to the PVY genome in aphids represented false positive as well (Table 1). These results are consistent with the fact that PLRV is transmitted by *M. persicae* in a nonpropagative manner (Day 1955; Eskandari 1979; Harrison 1958; Weidemann 2009). Furthermore, this result shows that gut clearing was effective at removing the majority of plant-derived sRNA from the aphid samples. The validity of our approach is confirmed by these controls, and enables us to observe novel sRNA interplay between the plant viruses and the aphid, as well as the

Fig. 1. Length distribution of small RNA (sRNA) reads generated in aphids, which aligned to the aphid genome. *Myzus persicae* aphids (~700 aphids/replicate, three to four replicates/treatment) were given a 72 h acquisition access period on *Potato leafroll virus* (PLRV)-infected plants, purified PLRV in 30% sucrose, *Potato virus Y* (PVY)-infected potato plants, 30% sucrose, or mock-inoculated potato plants, followed by 72 h gut clearing on turnip plants for all treatments. Aphid sRNA was extracted, sequenced, and analyzed as described in the methods. Values presented are the percentage of reads of that size aligning to the *M. persicae* genome, averaged across biological replicates. Error bars represent ± one standard error. Letters represent significantly different size distributions ($P < 0.05$) using multinomial distribution modeling.

relationship with the aphid obligate endosymbiont and an insect-infecting virus, as further described below.

PLRV, but not PVY, altered the size distribution of sRNA mapping to the aphid genome, including an increase in piwi-interacting RNA (piRNA). To test whether PVY and PLRV had an impact on the size distribution of *M. persicae*-derived sRNAs, we aligned the sRNA reads to the *M. persicae* genome. We found the distribution of *M. persicae*-derived sRNA to be distinct from all other treatments when aphids acquired PLRV, regardless of source (from infected plants or purified PLRV in sucrose diet, $P < 0.001$, Fig. 1). In aphids from the mock-inoculated potato treatment, a sucrose diet without PLRV, or PVY-infected plants, the most abundant size of siRNA was 22 nt. The 22 nt size may be the most abundant size for siRNAs produced in aphids, as this is also the dominant size produced by the cotton melon aphid, *Aphis gossypii* (Sattar et al. 2012). Fewer 22-nt siRNA matching the *M. persicae* genome were generated in aphids allowed an AAP on PLRV-infected plant tissue compared with those with an AAP on PVY-infected potato plants (Tukey's HSD test, $P = 0.0141$), mock-inoculated potato ($P = 0.0029$), and pure sucrose diet ($P = 0.0067$). Interestingly, this decreased number of 22-mers was also observed for

aphids feeding on purified PLRV (Tukey's HSD test, $P = 0.0147$, 0.0030, and 0.0070, Fig. 1). In a total of 20 pairwise comparisons for all treatments, aphids which had acquired PLRV from PLRV-infected plants or purified PLRV showed a significant increase in 26 and 27 nt sRNA (Tukey's HSD test, $P < 0.05$, Fig. 1), which is in the range of piRNAs (Luteijn and Ketting 2013). Over 90% of these 26- and 27-mer reads in our study were predicted to be piRNA (Supplementary Table S1) by analyzing them with the piRN deep learning algorithm (Wang et al. 2018). piRNA are often produced in the germ line (Luteijn and Ketting 2013). It is well known that feeding on PLRV-infected plants increases the fecundity of *M. persicae* (Castle and Berger 1993), a form of vector manipulation. Therefore, this observed increase in 26 to 27 mers may be the result of an increase in germ tissue, and as such, may serve as a molecular indicator of that vector manipulation by the virus. A nearly identical plasticity in the sRNA response of aphids has previously been observed in the aforementioned study on *A. gossypii* (Sattar et al. 2012). In that study, *A. gossypii* were exposed to melon lines expressing the virus aphid transmission (VAT) gene, which is a CS-NBS-LRR R gene and imparts resistance to melon against aphid feeding and several aphid-transmitted plant viruses. Aphids feeding on the VAT+ melon lines also produced an increase in 26- and 27-mers in the aphid (Sattar et al. 2012). Sattar and colleagues also provided evidence that this longer class of sRNA was enriched for piRNAs in *A. gossypii*. In their study, over 46% of the reads in this size class were enriched for transposable elements, a common feature of piRNA sequences in *Drosophila* and other animals (Brennecke et al. 2007). It is intriguing that the purified PLRV treatment in our study elicited a similar response in the aphid as feeding on plants expressing an antiviral, anti-aphid immunity R-gene. Collectively, these results suggest that plant viruses can prime the aphid's piRNA pathways through germ-line reprogramming in the absence of the plant in a manner similar to the expression of plant R-genes.

Exposure to the plant virus PLRV also altered the aphid's relationship with its obligate endosymbiont, *Buchnera aphidicola*. *B. aphidicola* contributes to the aphid's nutritional needs, providing the essential amino acids that the aphid is unable to synthesize or obtain from its diet in sufficient amounts (Hansen and Moran 2011). Differential expression of genes in amino acid biosynthesis pathways between bacteriocytes and other aphid tissues indicate complementarity between amino acid pathways encoded by the host and symbiont genomes (Hansen and Moran 2011). However, it is not known how *Buchnera* regulates gene expression, as *Buchnera* has lost most of the genes for transcriptional regulation that are present in free-living relatives, a feature common of endosymbiont genomes (Shigenobu et al. 2000). We found that about 9% of the sRNAs from aphid samples aligned to genes from *B. aphidicola* (Table 1), which was slightly more than what was reported in the Sattar et al. (2012) study. Among these *Buchnera* reads in our study, approximately 12% were derived from aminoacyl-tRNAs (Table 1), with asparagine being the most abundant aminoacyl-tRNA represented (Asn-tRNA). These reads are consistent with a novel class of sRNA, called tRNA fragments (tRFs), which have been found to be not only present, but abundant in both prokaryotes and eukaryotes (Sobala and Hutvagner 2011). The tRFs are products of precise tRNA processing rather than random degradation (Lee et al. 2009). They are often produced by nondicer nucleases and can be classified as 5', 3', and central fragments with respect to the mature or precursor tRNAs from which they are derived. In our data, the alignments of the sRNA sequences to the tRNAs were not random, but rather showed distinct distributions for each tRNA. For example, reads for Asn-tRNA predominantly aligned to the 5' end of the RNA and some to

Fig. 2. Small RNA (sRNA) mapping to the most abundant *Buchnera aphidicola* aminoacyl-tRNAs in aphid samples. *Myzus persicae* aphids (~700 aphids/replicate, three to four replicates/treatment) were given a 72 h acquisition access period on *Potato leafroll virus* (PLRV)-infected plants, purified PLRV in 30% sucrose, *Potato virus Y* (PVY)-infected potato plants, 30% sucrose, or mock-inoculated potato plants, followed by 72 h gut clearing on turnip plants for all treatments. Aphid sRNA was extracted, sequenced, and analyzed as described in the methods. Values presented as percent of the total *Buchnera*-derived sRNA generated in aphids, averaged across biological replicates. Shown are the abundance of sRNA reads mapping to the aminoacyl-tRNAs **A**, asparagine, **B**, tyrosine, **C**, threonine, **D**, methionine, **E**, serine, **F**, leucine, and **G**, glutamine, in descending order of abundance. Error bars represent $\pm$ one standard error. Letters represent significantly different treatments ($P < 0.05$).

the 3' end, Arg-tRNA had reads mapping to the middle of the RNA, and Leu-tRNA had reads mapping to the 3' end of the gene (Supplementary Fig. S3). The distribution of these reads along the mature tRNA did not change with treatment; only their relative abundance changed. The observation that these reads map to precise regions of the tRNA rather than being randomly dispersed leads us to believe that we are capturing actual tRNA-derived sRNA rather than degradation products of tRNAs. It is unknown, but possible, that the tRFs and sRNAs mapping to other *B. aphidicola* genes we detected represent a means of tRNA gene regulation in this endosymbiotic bacterium.

In other systems, tRFs were previously shown to perform regulatory roles and cleavage of tRNA is often induced by stressors, such as amino acid starvation (Thompson and Parker 2009). tRFs have even been found to associate with Argonaute proteins and may perform a silencing role similar to siRNAs or miRNAs (Sobala and Hutvagner 2011). In *Acyrtosiphon pisum* (the pea aphid), a significant correlation was reported between tRNA relative abundances and codon composition of *Buchnera* genes and tRNA abundances also changed during nutritional stress (Charles et al. 2006). In our data, the proportion of sRNA aligned to each *Buchnera* aminoacyl-tRNAs varied across treatments (Supplementary Table S2). Aphids on the sucrose diet treatment lacking PLRV and on PVY-infected potato produced significantly more sRNAs mapping to Asn-tRNA than aphids given an AAP on PLRV-infected potato plants (Fig. 2A). These data suggest that the amino acid requirements of aphids feeding on an unbalanced sucrose diet are similar to that of aphids given an AAP on PVY-infected potato and are in agreement with previous studies showed that aphids perceive a plant infected with a non-persistent virus as a low quality diet (Mauck et al. 2010, 2012). In contrast, for most of the other aminoacyl-tRNAs with an abundant number of reads, such as Ser, Leu, and Gln, the proportion of sRNAs found in aphids given an AAP on a PLRV source (either diet or infected tissue) was higher compared with aphids given an AAP on a sucrose diet lacking PLRV, mock-inoculated potato, or PVY-infected potato (Fig. 2). These data indicate that PLRV causes much greater

effects on the relative abundance of tRFs than PVY does, and may therefore have a greater effect on the relationship between the aphid vector and *B. aphidicola*.

PLRV altered the relationship of *M. persicae* with the insect-infecting virus MpDNV. In our aphid colony, MpDNV was visualized using FISH and detected along the entire alimentary canal including the posterior midgut, which is the site of PLRV acquisition (Supplementary Fig. S4). Qualitatively, no obvious change in virus distribution or abundance was observed between aphids given an AAP on uninfected or PLRV-infected plants. In other studies icosahedral particles of MpDNV were reported to localize in the cytoplasm of the aphid stomach cells but not the posterior midgut or hindgut cells (van Munster et al. 2003b). It is possible that different strains of MpDNV have different tropisms to regions of the gut in *M. persicae*.

Aphids, including *M. persicae*, encode homologs for all core genes of antiviral defense pathways, such as the Toll signaling, JAK-STAT, and viRNA pathways (Gerardo et al. 2010). The genes involved in the viRNA pathway, *dcr-2*, *ago-2*, and *r2d2*, are present in single copies in the pea aphid genome (Jaubert-Possamai et al. 2010). The machinery involved in the viRNA pathway has been shown to be functional via RNA interference in aphids and other insects (Jaubert-Possamai et al. 2007, 2010; Pitino et al. 2011; Sapountzis et al. 2014; Whyard et al. 2009). Thus, it was not surprising that several thousand sRNA reads from all aphid samples aligned to the genome of the insect-infecting MpDNV (GenBank accession number AY148187) (Table 1), with these viRNA ranging from 15 to 40 nt in length. The size distribution of viRNAs in aphids allowed an AAP on PLRV-infected potato plants was unique compared with other treatments ($P < 0.001$) (Fig. 3), with an abundance of unusually long sRNAs of 35 to 40 nt. To test whether the variance in the distribution could be derived from sRNAs within this unusual size range, a total of 36 pair-wise comparisons were performed for sRNAs in this size range for all treatments. All sRNAs in the 35 to 40 nt size range in aphids which had acquired PLRV from infected plants had a significantly higher relative

Fig. 3. Length distribution of small RNA (sRNA) reads generated in aphids, which aligned to the genome of the aphid virus *Myzus persicae densovirus* (MpDNV). *Myzus persicae* aphids (~700 aphids/replicate, three to four replicates/treatment) were given a 72 h acquisition access period on *Potato leafroll virus* (PLRV)-infected plants, purified PLRV in 30% sucrose, *Potato virus Y* (PVY)-infected potato plants, or mock-inoculated potato plants, followed by 72 h gut clearing on turnip plants for all treatments. Aphid sRNA was extracted, sequenced, and analyzed as described in the methods. Values presented are the percentage of reads of that size aligning to the MpDNV genome, averaged across biological replicates. Error bars represent $\pm$ one standard error. Letters represent significantly different size distributions ($P < 0.05$) using multinomial distribution modeling.

abundance compared with all other treatments (Tukey's HSD test, $P < 0.05$). These unusually long reads may be due to incomplete cleavage of viRNA precursors or may be other degradation products of viral transcripts. For most of the other treatments, the most abundant size of MpDNV viRNA reads was 22 nt, which is in the range of the most common size of sRNA generated by Dcr-2 in insects (Aliyari et al. 2008; Sabin et al. 2013; Xu et al. 2012). The number of 22 nt viRNAs was significantly lower in aphids given an AAP on PLRV-infected plants compared with mock-inoculated potato (Tukey's HSD test, $P = 0.0379$), similar to the reduction of 22-mer *M. persicae*-derived sRNA reported above for this same treatment. The distribution of viRNA was also distinct for aphids given an AAP on purified PLRV ($P < 0.001$) (Fig. 3) compared with all other treatments, for which the most abundant sizes were 17 and 22 nt, though this treatment did not exhibit the same unusually long sRNA as the aphids given an AAP on PLRV-infected plants, or less 22 mers compared with aphids given an AAP on mock-inoculated potato (Tukey's HSD, $P = 0.1977$).

Sequencing of *M. persicae* reveals integrations of densovirus-like sequences (DLSs) into the aphid genome (Clavijo et al. 2016). The integrated viral sequences have been shown to be transcribed in *M. persicae*, generating amino acid sequences that share 33 to 51% identity with MpDNV proteins (Clavijo et al. 2016). To check whether the sRNAs obtained in our experiments were in the sites of integration of MpDNV sequences into the *M. persicae* genome, we generated a consensus sequence for the MpDNV genome and aligned it to the *M. persicae* genome and found seven regions with

high similarity, based on an e-value cut-off of $<10^{-5}$. Four of these regions matched to the *M. persicae* genome with 100% similarity (Supplementary Table S3). We mapped the distribution of sRNA reads along the MpDNV genome and found no preference for these regions of integration (Supplementary Fig. S5). Since these regions with high similarity sequences to the aphid genome were not overrepresented in our sRNA dataset, it is likely that the sRNA reads mapping to MpDNV represent an actual antiviral response of aphids to MpDNV infection.

Considering that exposure to a PLRV-infected plant altered the aphid sRNA-mediated antiviral immune response, we wanted to test if PLRV infection in plants also altered MpDNV titer. MpDNV was quantified in single aphids allowed an AAP for 3 days on PLRV-infected and uninfected HNS plants (20 replicates/treatment). Aphids allowed an AAP on PLRV-infected HNS plants had significantly more copies of MpDNV than aphids placed on the uninfected HNS plants (Kruskal-Wallis test, $P = 0.02$, Fig. 4A). While we do not know if this increase in MpDNV titer in the aphid is a direct result of the aforementioned changes in the viRNA profile caused by exposure to PLRV-infected plants, these data suggest that PLRV causes two important changes to the aphid antiviral immune response against MpDNV.

In an attempt to identify which PLRV proteins were causing this effect on *M. persicae*, we tested the effect of P0 on MpDNV titer. P0 is the silencing suppressor protein encoded by PLRV and other virus in the genus *Polevirus*, that marks AGO1 for degradation in the plant host (Baumberger et al. 2007; Bortolamiol et al. 2007).

Fig. 4. Quantification of *Myzus persicae* densovirus (MpDNV) in *Myzus persicae* aphids. Aphid DNA was extracted and MpDNV titer was quantified via droplet digital PCR. **A**, Aphids fed on *Potato leafroll virus* (PLRV)-infected or uninfected hairy nightshade (HNS, *Solanum sarrachoides*) for 3 days (single aphids, 20 replicates per treatment). * indicates a P value less than 0.05 (Kruskal-Wallis test, $P = 0.02$). **B**, Aphids fed on *Nicotiana benthamiana* plants transiently expressing a PLRV infectious clone (PLRV), the viral silencing suppressor P0 driven by the 35S promoter (P0), the same 35S construct expressing GFP (GFP), or noninfiltrated (healthy) plants (4 to 8 aphids per replicate, 10 to 12 replicates per treatment) for 1 day (top) or 3 days (bottom). Letters show significantly different treatments ($P < 0.05$) via the Kruskal-Wallis test. **C**, Winged and nonwinged aphids (five aphids per replicate/16 replicates per treatment) collected from the same uninfected *P. floridana* colony. *** indicates a P value less than 0.001 (Mann-Whitney test, $P = 9.57 \times 10^{-5}$). Data are presented as copies of MpDNV found in 1 ng/ μ L of total aphid DNA. Boxes represent the interquartile range. The median is designated as a dark black line. Whiskers reach to maximum and minimum values. Circles are outliers.

Nonviruliferous (with no PLRV) aphids were placed on four different plant treatments for 1 day and 3 days: (i) *N. benthamiana* leaves transiently expressing the WT infectious clone of PLRV; (ii) leaves expressing only the PLRV P0 protein; (iii) leaves expressing a green fluorescent protein (GFP) control; and (iv) uninfected leaves (healthy control). No significant differences in MpDNV titer were found among the four treatments after 1 day (Fig. 4B, top). After 3 days (corresponding to 4 DPI), MpDNV titer was highest in aphids on the P0 treatment compared with all other treatments, including aphids fed on the PLRV-infected leaves, which would presumably also contain P0 expressed in the context of viral infection (Fig. 4B, bottom). There could be many reasons why expressing P0 alone in this experiment leads to an even greater increase in MpDNV than expressing the entire virus. It may be linked to the silencing suppressor activity of these particular constructs. Using a silencing suppressor activity assay (Shen et al. 2010) in *N. benthamiana* we showed that, 4 DPI, the same timepoint used in the aphid experiment, the silencing suppressor activity of P0 expressed alone was higher than P0 expressed in the context of WT PLRV (Supplementary Fig. S6). Regardless, these data show that expression of the PLRV silencing suppressor P0 in plants, even in the absence of a PLRV infection, induced higher MpDNV titers in the aphid. Thus, the P0 protein may be somehow altering the aphid antiviral immune system either directly in aphid cells or indirectly through the plant. Nothing is known as to whether P0 has a function in the aphid. These results raise additional questions about P0 activity in the plant and aphid during viral infection that are beyond the scope of the current study.

Aphids are polyphenic. In asexual aphid lineages, both winged and nonwinged individuals are produced. In the rosy apple aphid, *Dysaphis plantaginea*, densovirus (DpIDNV) infection induces the production of winged morphs (Ryabov et al. 2009). Densovirus-like sequences analogous to DpIDNV have been found to be integrated into the *M. persicae* genome, along with sequences from MpDNV (Clavijo et al. 2016). Considering the connection between DpIDNV and wing production in *D. plantaginea*, we quantified MpDNV titer in pools of winged and nonwinged *M. persicae* individuals. We found the difference in MpDNV titer in winged morphs and nonwinged morphs is highly significant, with winged morphs having a significantly higher MpDNV titer (Mann-Whitney test, $P < 0.001$) (Fig. 4C) and greater variance in titer (Levene's test, $P = 0.014$).

These data establish a correlation between MpDNV infection levels and wing production in this aphid species. Therefore, it is possible that MpDNV promotes wing production in *M. persicae* and this effect is mediated by PLRV to promote plant-to-plant virus dispersal. Gildow (1980) published a seminal paper showing that two aphid species, *Sitobion avenae* and *Rhopalosiphum padi*, reared on plants singly infected with three different species of yellow dwarf viruses (luteovirids) showed an increase in the production of winged morphs compared with aphids reared on uninfected plants. Furthermore, he showed that this increase was not related to the acquisition of the virus, but rather due to an unidentified component of the infected plant (Gildow 1980). Future work is needed to investigate the connection between MpDNV, wing production, and PLRV transmission.

Conclusion. These results showed intriguing ways in which the persistent plant virus PLRV alters sRNA-mediated processes in its vector, such as post-transcriptional gene regulation, the relationship with the obligate endosymbiont, and the relationship with an insect-infecting virus, whereas the nonpersistent PVY produced little or no effects on these same processes. However, one important limitation of this study is that the host switch of aphids from potato plants to turnip for gut-clearing during the sRNA-seq experiment may have had an effect on aphid sRNA production and metabolism, as

switches between solanaceous and brassicaeous hosts for *M. persicae* have previously been found to alter protein expression (Francis et al. 2006) and PLRV transmission (Pinheiro et al. 2017). However, the host switch to turnip was controlled for in all treatments so that conclusions can be drawn between the effect of the different virus treatments independent of any potential host switch effects on the sRNAs. Different potato cultivars were also used for aphid acquisition of PLRV versus PVY and may have induced some cultivar-specific changes in the aphids in these two treatments. Understanding the extent to which host switch plays a role in these interactions is an important area of investigation, especially considering how often host switch may occur in the field for a polyphagous vector such as *M. persicae*. Future experiments should also determine to what extent PLRV affects the aphid antiviral immune system in natural aphid populations, to control for any effects of the *M. persicae* genotype used in our study, and to better understand how aphid-infecting viruses may be used as aphid biocontrol agents.

ACKNOWLEDGMENTS

We thank J. Ingram (USDA-ARS, Ithaca, NY) for support with pesticide applications, N. Hickman (Boyce Thompson Institute, Ithaca, NY) for her assistance with planting and aphid handling, and A. Douglas and Heck lab members for productive discussion about these results.

LITERATURE CITED

- Aliyari, R., Wu, Q., and Li, H. W. 2008. Mechanism of induction and suppression of antiviral immunity directed by virus-derived small RNAs in *Drosophila*. *Cell Host Microbe* 4:387-397.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Alvarez, A. E., Garzo, E., Verbeek, M., Vosman, B., Dicke, M., and Tjallingii, W. F. 2007. Infection of potato plants with *Potato leafroll virus* changes attraction and feeding behaviour of *Myzus persicae*. *Entomol. Exp. Appl.* 125: 135-144.
- Baumann, P. 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* 59:155-189.
- Baumann, P., Baumann, L., Lai, C. Y., Rouhakhsh, D., Moran, N. A., and Clark, M. A. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: Intracellular symbionts of aphids. *Annu. Rev. Microbiol.* 49:55-94.
- Baumberger, N., Tsai, C. H., Lie, M., Havecker, E., and Baulcombe, D. C. 2007. The Pterovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. *Curr. Biol.* 17:1609-1614.
- Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., and Ziegler-Graff, V. 2007. The Pterovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. *Curr. Biol.* 17:1615-1621.
- Bouvaine, S., Boonham, N., and Douglas, A. E. 2011. Interactions between a luteovirus and the GroEL chaperonin protein of the symbiotic bacterium *Buchnera aphidicola* of aphids. *J. Gen. Virol.* 92:1467-1474.
- Brennecke, J., Aravin, A. A., and Stark, A. 2007. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128:1089-1103.
- Castle, S. J., and Berger, P. H. 1993. Rates of growth and increase of *Myzus persicae* on virus-infected potatoes according to type of virus-vector relationship. *Entomol. Exp. Appl.* 69:51-60.
- Castle, S. J., Mowry, T. M., and Berger, P. H. 1998. Differential settling by *Myzus persicae* (Homoptera: Aphididae) on various virus infected host plants. *Ann. Entomol. Soc. Am.* 91:661-667.
- Charles, H., Calevro, F., Vinuelas, J., Fayard, J. M., and Rahbe, Y. 2006. Codon usage bias and tRNA over-expression in *Buchnera aphidicola* after aromatic amino acid nutritional stress on its host *Acyrtosiphon pisum*. *Nucleic Acids Res.* 34:4583-4592.
- Chen, Y. R., Zheng, Y., Liu, B., Zhong, S., Giovannoni, J., and Fei, Z. 2012. A cost-effective method for Illumina small RNA-Seq library preparation using T4 RNA ligase 1 adenylated adapters. *Plant Methods* 8:41.

- Cilia, M., Tamborindeguy, C., Fish, T., Howe, K., Thannhauser, T. W., and Gray, S. 2011. Genetics coupled to quantitative intact proteomics links heritable aphid and endosymbiont protein expression to circulative polerovirus transmission. *J. Virol.* 85:2148-2166.
- Clavijo, G., Van Munster, M., Monsion, B., Bochet, N., and Brault, V. 2016. Transcription of densovirus endogenous sequences in the *Myzus persicae* genome. *J. Gen. Virol.* 97:1000-1009.
- Day, M. F. 1955. The Mechanism of the transmission of *Potato leafroll virus* by aphids. *Aust. J. Biol. Sci.* 8:498.
- DeBlasio, S. L., Johnson, R., and Mahoney, J. 2015. Insights into the polerovirus-plant interactome revealed by coimmunoprecipitation and mass spectrometry. *Mol. Plant-Microbe Interact.* 28:467-481.
- Ding, S. W., and Voinnet, O. 2007. Antiviral immunity directed by small RNAs. *Cell* 130:413-426.
- Eskandari, F. 1979. Evidence for lack of propagation of *Potato leafroll virus* in its aphid vector, *Myzus persicae*. *Phytopathology* 69:45.
- Feng, Y., Krueger, E. N., Liu, S., Dorman, K., Bonning, B. C., and Miller, W. A. 2017. Discovery of known and novel viral genomes in soybean aphid by deep sequencing. *Phytobiomes* 1:36-45.
- Francis, F., Gerkens, P., Harmel, N., Mazzucchelli, G., De Pauw, E., and Haubruge, E. 2006. Proteomics in *Myzus persicae*: Effect of aphid host plant switch. *Insect Biochem. Mol. Biol.* 36:219-227.
- Franco-Lara, L. F., McGeachy, K. D., Commandeur, U., Martin, R. R., Mayo, M. A., and Barker, H. 1999. Transformation of tobacco and potato with cDNA encoding the full-length genome of *Potato leafroll virus*: Evidence for a novel virus distribution and host effects on virus multiplication. *J. Gen. Virol.* 80: 2813-2822.
- Gerardo, N. M., Altincicek, B., and Anselme, C. 2010. Immunity and other defenses in pea aphids, *Acyrtosiphon pisum*. *Genome Biol.* 11:R21.
- Ghanim, M., Brumin, M., and Popovski, S. 2009. A simple, rapid and inexpensive method for localization of *Tomato yellow leaf curl virus* and *Potato leafroll virus* in plant and insect vectors. *J. Virol. Methods* 159: 311-314.
- Gildow, F., and D'arcy, C. 1990. Cytopathology and experimental host range of *Rhopalosiphum padi virus*, a small isometric RNA virus infecting cereal grain aphids. *J. Invertebr. Pathol.* 55:245-257.
- Gildow, F. E. 1980. Increased production of alatae by aphids reared on oats infected with *Barley yellow dwarf virus*. *Ann. Entomol. Soc. Am.* 73: 343-347.
- Gray, S., Cilia, M., and Ghanim, M. 2014. Circulative, "nonpropagative" virus transmission: an orchestra of virus-, insect-, and plant-derived instruments. *Adv. Virus Res.* 89:141-199.
- Guyomar, C., Legeai, F., Jousselin, E., Mougél, C., Lemaître, C., and Simon, J. C. 2018. Multi-scale characterization of symbiont diversity in the pea aphid complex through metagenomic approaches. *Microbiome* 6:181.
- Hansen, A. K., and Moran, N. A. 2011. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *Proc. Natl. Acad. Sci. USA* 108:2849-2854.
- Harrison, B. D. 1958. Studies on the behavior of *Potato leafroll* and other viruses in the body of their aphid vector *Myzus persicae* (Sulz.). *Virology* 6:265-277.
- Hodge, S., and Powell, G. 2008. Do plant viruses facilitate their aphid vectors by inducing symptoms that alter behavior and performance? *Environ. Entomol.* 37:1573-1581.
- Hodge, S., and Powell, G. 2010. Conditional facilitation of an aphid vector, *Acyrtosiphon pisum*, by the plant pathogen, *Pea enation mosaic virus*. *J. Insect Sci.* 10:155.
- Hwang, Y. T., Kalischuk, M., Fusaro, A. F., Waterhouse, P. M., and Kawchuk, L. 2013. Small RNA sequencing of *Potato leafroll virus*-infected plants reveals an additional subgenomic RNA encoding a sequence-specific RNA-binding protein. *Virology* 438:61-69.
- Ingwell, L. L., Eigenbrode, S. D., and Bosque-Perez, N. A. 2012. Plant viruses alter insect behavior to enhance their spread. *Sci. Rep.* 2:578.
- Jaubert-Possamai, S., Le Trionnaire, G., Bonhomme, J., Christophides, G. K., Rispé, C., and Tagu, D. 2007. Gene knockdown by RNAi in the pea aphid *Acyrtosiphon pisum*. *BMC Biotechnol.* 7:63.
- Jaubert-Possamai, S., Rispé, C., and Tanguy, S. 2010. Expansion of the miRNA pathway in the hemipteran insect *Acyrtosiphon pisum*. *Mol. Biol. Evol.* 27: 979-987.
- Jiu, M., Zhou, X. P., and Tong, L. 2007. Vector-virus mutualism accelerates population increase of an invasive whitefly. *PLoS One* 2:e182.
- Karasev, A. V., Hu, X., and Brown, C. J. 2011. Genetic diversity of the ordinary strain of *Potato virus Y* (PVY) and origin of recombinant PVY strains. *Phytopathology* 101:778-785.
- Kennedy, J. S., Day, M. F., and Eastop, V. F. 1962. A conspectus of aphids as vectors of plant viruses. Commonwealth Institute of Entomology, London.
- Kersch-Becker, M. F., and Thaler, J. S. 2014. Virus strains differentially induce plant susceptibility to aphid vectors and chewing herbivores. *Oecologia* 174: 883-892.
- Kliot, A., Cilia, M., Czosnek, H., and Ghanim, M. 2014. Implication of the bacterial endosymbiont *Rickettsia* spp. in interactions of the whitefly *Bemisia tabaci* with *Tomato yellow leaf curl virus*. *J. Virol.* 88:5652-5660.
- Kramer, M. 2014. Use of the posterior predictive distribution as a diagnostic tool for mixed models. Pages 82-101 in: Proceedings of the 26th Annual Kansas State University Conference on Applied Statistics in Agriculture. W. Song, ed. Manhattan, KS.
- Kutnjak, D., Rupa, M., Gutierrez-Aguirre, I., Curk, T., Kreuze, J. F., and Ravnika, M. 2015. Deep sequencing of virus-derived small interfering RNAs and RNA from viral particles shows highly similar mutational landscapes of a plant virus population. *J. Virol.* 89:4760-4769.
- Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25.
- Lee, Y. S., Shibata, Y., Malhotra, A., and Dutta, A. 2009. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). *Genes Dev.* 23: 2639-2649.
- Li, F., and Ding, S. W. 2006. Virus counterdefense: Diverse strategies for evading the RNA-silencing immunity. *Annu. Rev. Microbiol.* 60:503-531.
- Li, H., Handsaker, B., and Wysoker, A. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* 25:2078-2079.
- Li, R., Gao, S., Hernandez, A. G., Wechter, W. P., Fei, Z., and Ling, K. S. 2012. Deep sequencing of small RNAs in tomato for virus and viroid identification and strain differentiation. *PLoS One* 7:e37127.
- Luteijn, M. J., and Ketting, R. F. 2013. PIWI-interacting RNAs: From generation to transgenerational epigenetics. *Nat. Rev. Genet.* 14:523-534.
- MacKinnon, J. P. 1961. Preference of aphids for excised leaves to whole plants. *Can. J. Zool.* 39:445-447.
- Mauck, K., Bosque-Pérez, N. A., Eigenbrode, S. D., De Moraes, C. M., Mescher, M. C., and Fox, C. 2012. Transmission mechanisms shape pathogen effects on host-vector interactions: Evidence from plant viruses. *Funct. Ecol.* 26: 1162-1175.
- Mauck, K. E., De Moraes, C. M., and Mescher, M. C. 2010. Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proc. Natl. Acad. Sci. USA* 107:3600-3605.
- Milne, I., Stephen, G., and Bayer, M. 2013. Using Tablet for visual exploration of second-generation sequencing data. *Brief. Bioinform.* 14:193-202.
- Mlotshwa, S., Pruss, G. J., and Vance, V. 2008. Small RNAs in viral infection and host defense. *Trends Plant Sci.* 13:375-382.
- Moon, J. S., Domier, L. L., Mccoppin, N. K., D'arcy, C. J., and Jin, H. 1998. Nucleotide sequence analysis shows that *Rhopalosiphum padi virus* is a member of a novel group of insect-infecting RNA viruses. *Virology* 243: 54-65.
- Mutti, N. S., Park, Y., Reese, J. C., and Reece, G. R. 2006. RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrtosiphon pisum*. *J. Insect Sci.* 6:1-7.
- Nault, L. R. 1997. Arthropod transmission of plant viruses: A new synthesis. *Ann. Entomol. Soc. Am.* 90:521-541.
- Ng, J. C., and Falk, B. W. 2006. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annu. Rev. Phytopathol.* 44:183-212.
- Pickett, J. A., Wadhams, L. J., Woodcock, C. M., and Hardie, J. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67-90.
- Pinheiro, P. V., Ghanim, M., and Alexander, M. 2017. Host plants indirectly influence plant virus transmission by altering gut cysteine protease activity of aphid vectors. *Mol. Cell. Proteomics* :S230-S243.
- Pitino, M., Coleman, A. D., Maffei, M. E., Ridout, C. J., and Hogenhout, S. A. 2011. Silencing of aphid genes by dsRNA feeding from plants. *PLoS One* 6: e25709.
- Poliakov, A., Russell, C. W., Ponnala, L., Hoops, H. J., Sun, Q., Douglas, A. E., and van Wijk, K. J. 2011. Large-scale label-free quantitative proteomics of the pea aphid-*Buchnera* symbiosis. *Mol. Cell. Proteomics* 10: M110.007039.
- Quast, C., Pruesse, E., and Yilmaz, P. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41:D590-D596.
- R Core Team. 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

- Ryabov, E. V. 2007. A novel virus isolated from the aphid *Brevicoryne brassicae* with similarity to Hymenoptera picorna-like viruses. *J. Gen. Virol.* 88: 2590-2595.
- Ryabov, E. V., Keane, G., Naish, N., Evered, C., and Winstanley, D. 2009. Densovirus induces winged morphs in asexual clones of the rosy apple aphid, *Dysaphis plantaginea*. *Proc. Natl. Acad. Sci. USA* 106:8465-8470.
- Sabin, L. R., Zheng, Q., and Thekkat, P. 2013. Dicer-2 processes diverse viral RNA species. *PLoS One* 8:e55458.
- Sapountzis, P., Duport, G., and Balmand, S. 2014. New insight into the RNA interference response against cathepsin-L gene in the pea aphid, *Acyrtosiphon pisum*: Molting or gut phenotypes specifically induced by injection or feeding treatments. *Insect Biochem. Mol. Biol.* 51:20-32.
- Sattar, S., Addo-Quaye, C., Song, Y., Anstead, J. A., Sunkar, R., and Thompson, G. A. 2012. Expression of small RNA in *Aphis gossypii* and its potential role in the resistance interaction with melon. *PLoS One* 7:e48579.
- Shen, M., Xu, Y., Jia, R., Zhou, X., and Ye, K. 2010. Size-independent and noncooperative recognition of dsRNA by the *Rice stripe virus* RNA silencing suppressor NS3. *J. Mol. Biol.* 404:665-679.
- Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* 407:81-86.
- Sobala, A., and Hutvagner, G. 2011. Transfer RNA-derived fragments: Origins, processing, and functions. *Wiley Interdiscip. Rev. RNA* 2:853-862.
- Teixeira, M., Sela, N., and Ng, J. 2016. A novel virus from *Macrosiphum euphorbiae* with similarities to members of the family *Flaviviridae*. *J. Gen. Virol.* 97:1261-1271.
- Thompson, D. M., and Parker, R. 2009. Stressing out over tRNA cleavage. *Cell* 138:215-219.
- van Der Wilk, F., Dulleman, A. M., Verbeek, M., and Van Den Heuvel, M. J. F. J. 1997. Nucleotide sequence and genomic organization of *Acyrtosiphon pisum* virus. *Virol. J.* 238:353-362.
- van Munster, M., Dulleman, A. M., and Verbeek, M. 2002. Sequence analysis and genomic organization of *Aphid lethal paralysis virus*: A new member of the family *Dicistroviridae*. *J. Gen. Virol.* 83:3131-3138.
- van Munster, M., Dulleman, A. M., and Verbeek, M. 2003a. A new virus infecting *Myzus persicae* has a genome organization similar to the species of the genus *Densovirus*. *J. Gen. Virol.* 84:165-172.
- van Munster, M., Dulleman, A. M., and Verbeek, M. 2003b. Characterization of a new densovirus infecting the green peach aphid *Myzus persicae*. *J. Invertebr. Pathol.* 84:6-14.
- Wang, K., Hoeksema, J., and Liang, C. 2018. piRNN: Deep learning algorithm for piRNA prediction. *PeerJ* 6:e5429.
- Weidemann, H.-L. 2009. Zur Vermehrung des Kartoffelblattrollvirus in der Blattlaus *Myzus persicae* (Sulz.). *J. Appl. Entomol.* 94:321-330.
- Whyard, S., Singh, A. D., and Wong, S. 2009. Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* 39: 824-832.
- Williamson, C., Rybicki, E., Kasdorf, G., and Von Wechmar, M. 1988. Characterization of a new picorna-like virus isolated from aphids. *J. Gen. Virol.* 69:787-795.
- Xu, Y., Huang, L., Fu, S., Wu, J., and Zhou, X. 2012. Population diversity of *Rice stripe virus*-derived siRNAs in three different hosts and RNAi-based antiviral immunity in *Laodelphax striatellus*. *PLoS One* 7:e46238.
- Zheng, Y., Gao, S., and Padmanabhan, C. 2017. VirusDetect: An automated pipeline for efficient virus discovery using deep sequencing of small RNAs. *Virology* 500:130-138.