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GENETIC DIVERSITY AND EVOLUTION

Host Range and Population Survey of *Spodoptera frugiperda* Rhabdovirus

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ABSTRACT The Sf9 and Sf21 cell lines derived from ovarian tissues of the wide-host-range phytophagous lepidopteran *Spodoptera frugiperda* are widely used for research and commercial-scale production of recombinant proteins. These cell lines are chronically infected with a rhabdovirus (Sf-RV) that does not cause any overt cytopathic effects. We demonstrate that wild populations of *S. frugiperda* in the eastern United States and Caribbean are infected with genetically diverse strains of Sf-RV and that this virus is also capable of infecting cells of *Spodoptera exigua*, *Heliothis subflexa*, and *Bombyx mori*. Feeding studies demonstrated the ability of *S. frugiperda* larvae to deposit Sf-RV onto human-consumed vegetables during feeding. Although no evidence for replication in two species of plant cells was detected, subcellular localization studies demonstrated that the Sf-RV nucleocapsid was targeted to plasmodesmata, while two forms of the accessory protein were differentiated on the basis of their ability to localize to nuclei. Collectively, the results from this study suggest that environmental exposure of humans to Sf-RV is likely to be commonplace and frequent, but its inability to replicate in plant or human cells suggests that there is no substantial risk to human health.

IMPORTANCE Insect-derived cell lines are widely used commercially for the production of vaccines and protein-based pharmaceuticals. After decades of safe and beneficial use, it was a surprise to the biotechnology industry to discover an endemic rhabdovirus in Sf9 cells. This discovery was made possible only by the substantial advancements in DNA sequencing technologies. Given the public health concerns associated with many rhabdovirus species, several initiatives were undertaken to establish that *Spodoptera frugiperda* rhabdovirus (Sf-RV) does not pose a threat to humans. Such actions include the generation of cell lines that have been cleared of Sf-RV. Given that Sf9 is derived from a moth whose larvae feed on human-edible foods, we explored the prevalence of Sf-RV in its wild and lab-grown populations, as well as its ability to be deposited on food items during feeding. Collectively, our data suggest that there is no overt risk from exposure to Sf-RV.

KEYWORDS *Nicotiana benthamiana*, Sf21, Sf9, human-edible foods, population structure

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Spodoptera frugiperda, the fall armyworm, is an extremely important agricultural pest. This polyphagous insect has a host range of more than 80 plants, many of which produce human-consumed foods such as sweet corn, tomatoes, strawberries, apples, and peanuts (1–4). In addition to *S. frugiperda*, at least nine *Spodoptera* species are native to the southern United States, where many food crops are grown. These species include Southern armyworm *S. eridania* (Stoll), and the beet armyworm *S. exigua* (Hübner) (5). Given their tropical origin, *Spodoptera* pests in the United States are restricted to overwintering in an area spanning from Texas to Florida. However, during the summer months, in some years, populations of these insects can rapidly expand

their ranges as far as Canadian provinces (6). Effective control of *S. frugiperda*, particularly in corn and cotton, can be achieved through the use of transgenic plants expressing Cry proteins from *Bacillus thuringiensis*, to which resistance can be selected in areas of continuous use (7–9).

In addition to its significant impacts on food and fiber production, *S. frugiperda*, as with other lepidopterans such as *Trichoplusia ni*, is linked to a wide range of biotechnologies through the use of cell lines used for research and commercial-scale production of vaccines, biobetters, and other recombinant proteins (10–14). In particular, the Sf21 and Sf9 cell lines derived from ovarian tissues of *S. frugiperda* are used extensively in combination with baculovirus-derived vectors for recombinant protein production. The Sf9 and Sf21 cell lines have been used safely for human pharmaceutical production for a decade, and no known adventitious viruses had been found to be capable of infecting them. Recently, however, a novel rhabdovirus has been identified in all Sf9 cell lines, now called Sf-rhabdovirus (here, Sf-RV) (15–20). The discovery of Sf-RV was reported by Ma et al. (15) based on work performed at the U.S. FDA. The virus was also independently discovered by Takeda Vaccines, Inc. (then LigoCyte Pharmaceuticals) in 2012 via cloning and sequencing efforts that were intended to screen for the presence of novel insect retrovirus sequences in the Sf9 cell line. Sf-RV is noncytopathic in Sf9 cells and has a 13.5-kb negative-sense single-stranded RNA genome characteristic of viruses in the *Mononegavirales* (15). The genome is organized into six open reading frames (ORFs) in the order nucleocapsid protein (N), phosphoprotein (P), matrix protein, (M), glycoprotein (G), accessory protein of unknown function (F, alternatively referred to as X), and RNA-dependent RNA polymerase (L), flanked by noncoding leader and trailer regions (15). Subsequent studies have shown that the F protein is dispensable for infectivity in Sf9 cells, and deletion of 320 nucleotides from this ORF can occur when Sf9 cells are continuously passaged (16–18, 20).

To reduce the potential risk of exposure to Sf-RV, cell lines cleared of the Sf-RV have been produced, which have been shown to be of equivalent utility to Sf-RV-infected lines (17–19). However, as this study demonstrates, the environmental presence of Sf-RV and the likelihood for regular human exposure argues that there are no known human health risks warranting the need to change Sf-9 cell lines currently used in commercial applications. Importantly, Sf-RV has been shown to be noninfectious in human, mammalian, and nonlepidopteran (*Drosophila*-derived) cell lines (15, 20). These studies suggest that Sf-RV and the continued use of Sf-RV-infected lines already in commercial production systems do not pose adverse risks to human health. Further assurance that Sf-RV is not a significant threat to human health would be provided if there was a greater understanding of the human exposure to Sf-RV as a result of widespread occurrence in wild *Spodoptera* populations and if these insects have the ability to deposit this virus on food for human consumption. As such, establishing that humans have long been exposed to Sf-RV via routes of insect feeding is an important criterion in the evaluation of the risk to humans. Furthermore, given the polyphagous nature of *S. frugiperda*, it is important to know if plants can provide reservoirs for this virus or serve to amplify it when inoculated. To address these concerns, we surveyed wild-caught *S. frugiperda* from populations in seven southeastern states in the United States and in the Dominican Republic. Additionally, we examined the ability to detect Sf-RV RNA on vegetables after insect feeding and the potential for Sf-RV replication in other insect and plant cells. Finally, we determined the full-length genomes of Sf-RV that we identified in two additional insect cell lines from phytophagous species, *Heliothis subflexa* and *Bombyx mori*. Taken together, we show that Sf-RV is widely distributed in nature and has the ability to replicate in additional lepidopteran cell lines from insect species that possess the potential to deposit virus on food plants. Additionally, it was shown previously that this highly labile virus is not infectious in mammalian cells, and we demonstrate here its inability to replicate in plant cells, in which it exhibits protein localization patterns similar to those of plant-adapted species. Though further investigations are warranted, the present studies suggest that regular

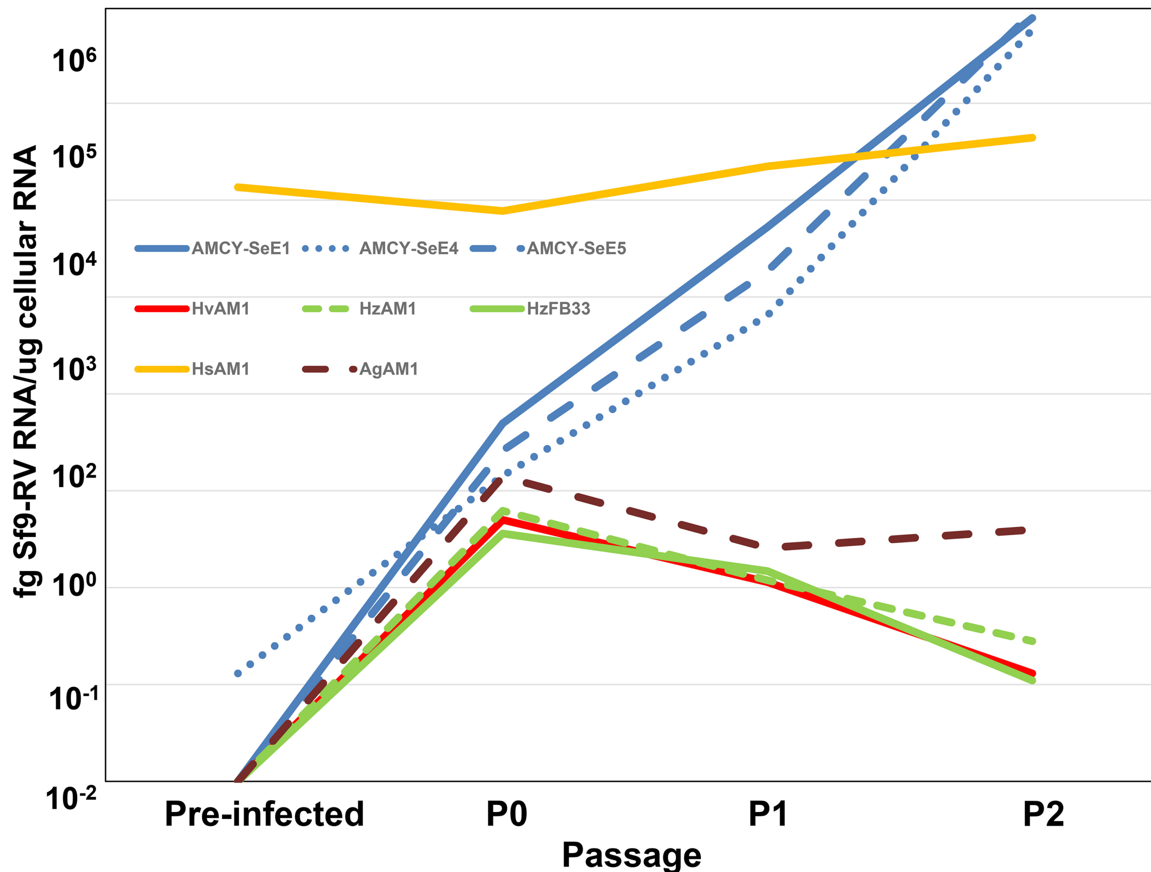

FIG 1 Detection of Sf9 rhabdovirus RNA in insect cells exposed to Sf9 cell-conditioned medium. Cell lines obtained from the Agricultural Research Service (ARS) of the U.S. Department of Agriculture (USDA) were inoculated with Sf9 cell-conditioned medium and passaged three times at intervals of 72 h (P0, P1, and P2). Cell lines derived from *Spodoptera exigua* eggs (blue lines) were highly susceptible to Sf-RV. The ovary-derived *Heliothis subflexa* cell line (yellow line) was found to be endemically infected with Sf-RV at the start of the experiment (preinfected). The *Helicoverpa zea* ovary (dashed green line) or *H. zea* fat body-derived (solid green line) and *H. virescens* ovary (red line) cell lines did not support replication of Sf-RV.

environmental exposure to Sf-RV is likely significant but represents negligible risk to human health.

RESULTS

Infectivity of Sf-RV in insect cell lines. The ubiquitous presence of Sf-RV in Sf9 cells, its ability to reinfect these cells (20), and the propensity of *Spodoptera* sp. to exist in mixed populations raised the possibility that Sf-RV may infect or be endemic to other phytophagous lepidopterans (1, 9, 21). Therefore, we screened cell lines derived from *S. exigua*, *Heliothis virescens*, *Helicoverpa zea*, *H. subflexa*, and *Anticarsia gemmatalis* for Sf-RV infectivity by exposing them to filtered supernatants of Sf9 cultures. The accumulation of Sf-RV RNA was monitored over two passages by reverse transcriptase PCR (RT-PCR). Surprisingly, *H. subflexa* cells were positive for Sf-RV without prior addition of medium from Sf9 cultures, demonstrating that they were likely already infected with Sf-RV. The titer of Sf-RV in *H. subflexa* cells remained high at ~10^4 fg Sf-RV RNA/μg cell RNA (Fig. 1). *H. virescens*, *H. zea*, and *A. gemmatalis* cells exhibited no propensity to host Sf-RV, with a rapid decline of detectable virus RNA after a single passage. In contrast, Sf-RV titers in *S. exigua* cell lines rose from undetectable to two orders of magnitude higher than those in *H. subflexa* after two passages. These observations demonstrated that Sf-RV has a host cell range beyond *S. frugiperda* and provided a new host cell platform (*S. exigua*) in which to further investigate Sf-RV infectivity. Also, it should be noted that Sf-RV infectivity in *S. exigua* cells does not require the presence of the intact

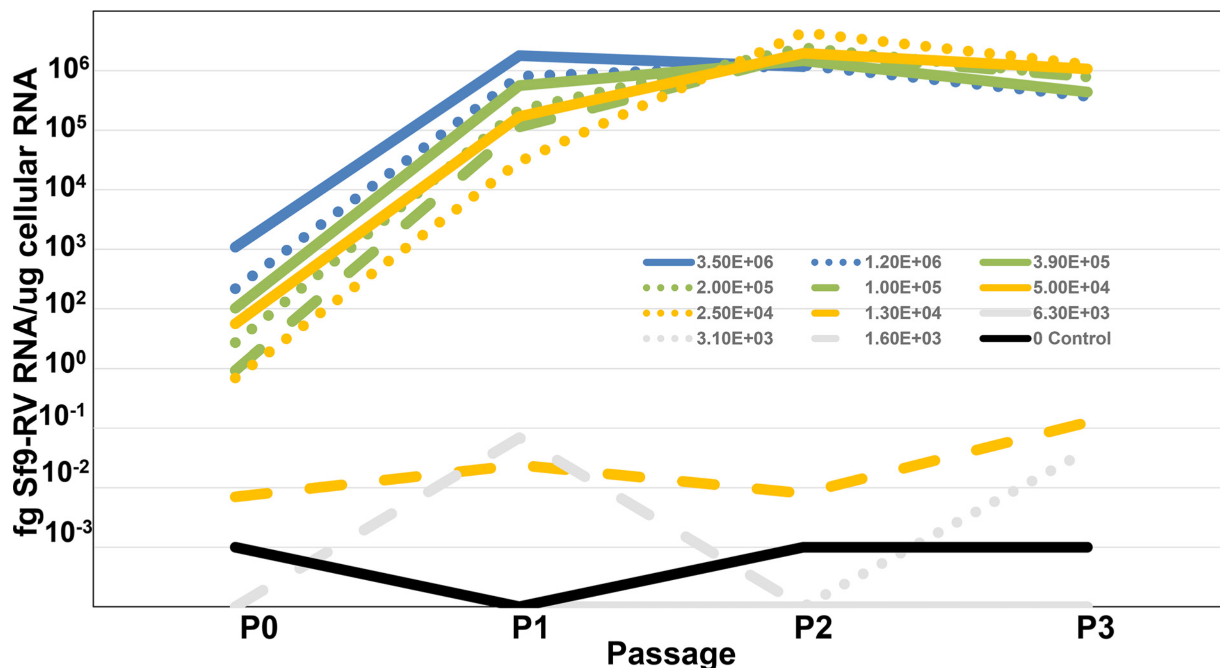

FIG 2 Infectivity of Sf-RV in Sf9 cells as a function of virus titer. Sf9 cell supernatants containing 10^6 (blue lines), 10^5 (green lines), 10^4 (yellow lines), 10^3 (gray lines), or no (black line) particles of Sf-RV.

accessory gene, since the Sf-RV found in the Takeda Sf9 cell line contains the same 320-nucleotide deletion noted by others (16–18, 20).

Titrations of Sf-RV infectivity on *S. exigua* cells. The experimental results presented in Fig. 2 are derived from a classic virus titration experiment (22) in which infectious virus in Sf9 cell lysates was quantified by inoculating susceptible *S. exigua* cells with decreasing quantities of virus-containing lysate and monitoring the inoculated cultures for evidence of Sf-RV infectivity by PCR. As in all infectivity studies, there will always be a greater number of total virus particles (quantified by genomic RNA copy number) than infectious particles. Our conclusions about this ratio suggest that the observation that there are many more copies of viral genomic RNA than of infectious Sf-RV viral particles is not unusual for rhabdoviruses. While the infectious-to-noninfectious particle ratio was low, it is important to point out that Sf-RV infectivity in *S. exigua* cells was quite robust, in that once an infection was detected, it accelerated rapidly to equilibrium.

Based on the observation that Sf-RV is capable of replication in *Spodoptera exigua* cells, two experiments were completed in which Sf-RV contained within culture supernatants of Sf9 cells was titrated for infectivity on the *S. exigua* cell line SeE1 to determine the ratio of infectious units to noninfectious rhabdovirus particles. The two Sf9 cell supernatants used for the titrations on SeE1 cells contained 3.5×10^7 and 5.4×10^7 particles per ml, as shown by RT-PCR. This determination was performed by quantitative RT-PCR specific for minus strand genomic RNA and following RNase treatment to eliminate any free nonencapsulated rhabdovirus RNA. Sequential flasks of SeE1 cells were inoculated with 3-fold serial dilutions of the Sf9 cell supernatants. Following inoculation, each flask was incubated until confluent (passage 0) and then passaged an additional 3 or 4 times at a 1:10 dilution. Cells not transferred into the next passage were subjected to total RNA purification. Purified RNA from each flask at each passage was subjected to RT-PCR analysis for the detection and quantification of the Sf9 rhabdovirus to determine the highest dilution that still contained at least one infectious unit. Figure 2 shows the results of the two titrations in which similar results were obtained, indicating an infectious titer of approximately 2×10^3 infectious units per ml. Based on the genomic RNA copy numbers in the original supernatants, this

resulted in infectious-to-noninfectious particle ratios of approximately 1:17,000 and 1:26,000 in the two experiments. It is important to note that infectivity progressed rapidly in those SeE1 cell flasks that became infected and that infection was obvious by passage 1 in all but one flask in which infection was evident by passage 2.

Presence of Sf-RV in wild populations of *S. frugiperda*. As both the Sf9 and Sf21 cell lines were derived from ovarian tissue from an *S. frugiperda* insect specimen, we determined whether the Sf9 rhabdovirus could be detected at a significant frequency in other wild or laboratory colony specimens of *S. frugiperda*. To determine the extent of the presence of this virus in the environment, we tested a number of adult *S. frugiperda* specimens captured in pheromone traps around the eastern region of the United States, Dominican Republic, and Puerto Rico from surveys conducted over multiple years by the USDA-ARS Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, FL. Table 1 shows a list of 67 insect specimens that were collected and processed for RNA and successfully tested for the rhabdovirus by RT-PCR with appropriate negative controls. A number of the moth RNA samples exhibited very strong rhabdovirus signals (shaded in Table 1) for both the L gene and the accessory gene, providing strong evidence that these insects were infected with the same virus present in Sf9 cells. It is important to note that the accessory gene PCR assay targets a sequence that is not present in viruses containing the 320-nucleotide deletion. In the L gene assay, 54 of 67 samples (80.6%) were at least weakly positive, with 13 samples being negative (cycle threshold [C_T] of ≥40). In the accessory gene assay, all 67 samples (100%) were positive, with C_T values ranging from 35.85 to 14.89. For most of the moth RNA samples, the accessory gene assay was more sensitive than the L gene assay, which likely accounts for the greater number of positive samples.

Variation in N gene sequences from geographically distant moth samples. The major factor contributing to the wide variance in the ability to detect rhabdovirus signals in screens of wild-caught moths was likely due to their states of decay prior to storage and RNA isolation. That said, it is noteworthy that the accessory gene was present in every moth RNA sample collected from the environment, even though the accessory gene PCR primer and probe set used in the study targeted a sequence that is absent in the Takeda Sf-RV and which has been found to be unstable in the Sf-RV found in Sf9 cells derived from the ATCC during continuous passage (data not shown). Fortunately, 9 of the 67 moth RNA samples listed in Table 1 yielded strong RT-PCR signals, which made cloning and sequence determination of the full-length N ORF possible. These data were used to generate a phylogenetic comparison of this gene in the wild population with that of Sf-RV found in Sf9 cell lines (Fig. 3). We focused on the N gene for downstream analyses, as this gene is typically used to assess population structure in negative-strand RNA viruses (23–25). Sequence homologies between the field specimens and the published Sf9 rhabdovirus sequence from Sf9 cells ranged from 94% to 99%. The N gene sequences clustered into two distinct clades, suggesting subspecies structure in the Sf-RV population as has been noted for other negative-strand RNA viruses (26).

Sequence analysis of Sf-RV found in other insect cells. Screening a variety of insect cell lines showed that Sf-RV is constitutively present in the *H. subflexa* cell line HsAM1, based on PCR data. The identification of Sf-RV in an insect line from a genus other than *Spodoptera* suggested that this virus might be capable of growth in a variety of insect species and be significantly more widespread in insects other than *S. frugiperda* in the environment. Therefore, we sequenced the version of Sf-RV found in the HsAM1 cell line to determine if it was indeed the same virus. In addition to *H. subflexa*, a GenBank database entry (AK377209.1) for an mRNA sequence clone derived from *Bombyx mori* was found to be essentially identical to one of the genes in the Sf-RV genome, implying that this virus can also be found in silk worm insects or cell lines. Indeed, acquisition of the *B. mori* cell line Bm-N and analysis by PCR indicated that this cell line was infected with Sf-RV similarly to the *H. subflexa* HsAM1 cell line. Complete sequence contigs were generated for Sf-RVs found in both the HsAM1 and Bm-N cell

TABLE 1 Detection of Sf-RV in wild-caught *S. frugiperda* from counties in American states and Caribbean countries[a]

				C_T	
Sample no.	**Yr**	**State**	**County**	**L gene**	**Accessory gene**
1	2011	AL	Conecuh	37.92	33.57
2	2011	AL	Conecuh	24.6	17.25
3	2011	AL	Conecuh	38.04	31.37
4	2015	DR	Santo Domingo	37.14	34.55
5	2010	FL	Alachua	34.82	33.93
6	2014	FL	Alachua	>40.00	34.27
7	2011	FL	Gadsden	>40.00	32.14
8	2013	FL	Suwannee	35.06	35.13
9	2014	FL	Miami-Dade	>40.00	32.09
10	2012	FL	Hardee	38.98	32.92
11	2012	FL	Hillsborough	35.5	33.25
12	2010	FL	Levy	37.13	34.83
13	2012	GA	Hart	39.94	33.92
14	2014	GA	Spalding	21.57	24.62
15	2014	IL	Warren	15.96	15.8
16	2010	IA	Story	31.06	30.59
17	2012	KY	Caldwell	17.36	16.62
18	2014	SC	Charleston	33.53	27.95
19	2012	TX	Nueces	32.08	31.29
23	2011	AL	Conecuh	>40.00	33.98
24	2011	AL	Conecuh	23.7	16.61
25	2011	AL	Conecuh	34.91	32.66
26	2011	AL	Conecuh	>40.00	31.14
27	2011	AL	Conecuh	38.01	30.03
28	2011	AL	Conecuh	37.99	32.22
29	2011	FL	Gadsden	>40.00	30.78
30	2012	FL	Hardee	39.41	32.91
31	2012	FL	Hillsborough	21.45	22.32
32	2013	FL	Suwannee	38.01	32.31
33	2014	FL	Alachua	21.85	23.46
34	2014	FL	Miami-Dade	>40.00	34.07
35	2012	GA	Hart	>40.00	31.49
36	2010	IA	Story	34.45	29.36
37	2014	IL	Warren	>40.00	33.57
38	2012	TX	Nueces	37.54	32.55
39	2014	SC	Charleston	22.14	21.97
40	2012	KY	Caldwell	35.43	32.78
41	2014	GA	Spalding	20.99	23.23
42	2010	FL	Levy	37.66	30.72
43	2014	FL	Miami-Dade	37.08	32.16
44	2014	FL	Alachua	>40.00	31.36
45	2010	FL	Alachua	34.89	32.11
46	2011	AL	Conecuh	39.01	30.23
47	2011	AL	Conecuh	37.9	30.61
48	2015	DR	Santo Domingo	37.06	32.61
49	2011	FL	Gadsden	>40.00	34.04
50	2012	FL	Hillsborough	37.7	32.04
51	2011	AL	Conecuh	>40.00	32.94
52	2011	AL	Conecuh	36.58	29.09
53	2011	AL	Conecuh	38.58	33.14
54	2011	AL	Conecuh	37.81	35.85
55	2011	AL	Conecuh	37.3	30.63
56	2012	TX	Nueces	35.4	31.67
57	2014	SC	Charleston	34.6	31.67
58	2010	FL	Alachua	38.58	30.37
59	2011	FL	Gadsden	>40.00	31.95
60	2012	FL	Hardee	17.58	14.89
61	2010	IA	Story	34.02	25.5
62	2014	IL	Warren	34.68	28.19
63	2013	FL	Suwannee	35.84	27.34
64	2014	FL	Miami-Dade	34.18	29.36
65	2014	FL	Alachua	39.35	29.19
66	2014	GA	Spalding	38.75	33.18

(Continued on next page)

TABLE 1 (Continued)

Sample no.	Yr	State	County	C_T L gene	C_T Accessory gene
67	2011	AL	Conecuh	32.66	31.16
68	2010	FL	Alachua	37.28	32.24
69	2011	FL	Gadsden	24.19	26.71
70	2012	FL	Hillsborough	35.88	31.07
81	2012	FL	Hardee	24.6	22.32

[a]Shading indicates samples that yielded full-length N gene PCR amplicons used for phylogenetic tree construction.

lines. In Table 2, the predicted proteins encoded by Sf-RV variants are compared. Notably, the size of the accessory protein in the *H. subflexa* strain is reduced from 12.63 kDa to 8.95 kDa due to a 320-bp deletion in the coding region of this gene. The sequence contig determined for Sf-RV from *B. mori* confirms the presence of the intact accessory gene region.

Detection of Sf-RV virus RNA on vegetables following *S. frugiperda* larva feeding. Since *S. frugiperda* insects are widespread in parts of North and South America, and more recently, in Africa, where they feed on important human food crops, it was of interest to determine if *S. frugiperda* larvae could potentially deposit Sf-RV on human food plants while feeding. To this end, *S. frugiperda* eggs were acquired from a commercial vendor and allowed to hatch into larvae and feed on 13 different vegetables in the laboratory for 18 h. Prior to the initiation of this study, we confirmed the presence of Sf-RV in 100% of larvae hatched from the commercially acquired eggs. Following feeding, vegetable specimens were harvested and subjected to RNA isolation, and purified RNA was tested for the presence of Sf-RV RNA. Thirty-seven of forty-two vegetable samples were shown to be positive for the presence of Sf-RV RNA, while all negative-control vegetables not exposed to *S. frugiperda* larvae were found to be negative. These data are shown in Fig. 4, in which 37 of 42 (88%) vegetable samples were positive (C_T value, <35) for the presence of Sf-RV RNA and a few of the samples had very high titers (C_T value, <25). These data are consistent with the possibility that *S. frugiperda* larvae deposit infectious virus on vegetables during feeding and that humans might be exposed to Sf-RV via raw vegetable handling and/or consumption. In

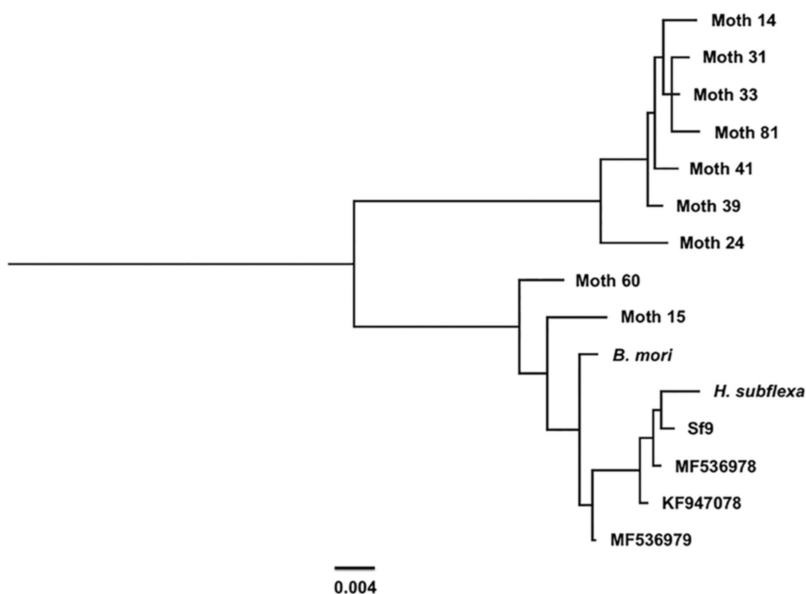

FIG 3 Phylogeny of N gene sequences from wild populations of *S. frugiperda* and insect cell lines. Scale bar, 0.004 substitutions per site.

TABLE 2 Variation in the proteins encoded by three strains of Sf-RV isolated from different species

Strain	ORF	Function	MW[a]	pI	NLS[a]
Sf-RV (Takeda)	N	Nucleocapsid	60.85	5.10	
	P	Phosphoprotein	41.61	6.59	^{318}RKPR^{323}
	M	Matrix protein	34.97	9.53	
	G	Glycoprotein	69.90	8.38	
	X	Unknown	**8.95**	5.80	
	L	Polymerase	24.45	8.23	^{9}RKKRP^{15}, ^{554}KKRH^{559}
H. subflexa	N	Nucleocapsid	60.78	5.00	
	P	Phosphoprotein	42.05	6.82	^{321}RKPR^{326}
	M	Matrix protein	34.94	9.57	
	G	Glycoprotein	70.18	8.52	^{329}RHKR^{323}
	X	Unknown	**8.95**	5.80	
	L	Polymerase	24.41	8.01	^{9}RKKRP^{15}, ^{554}KKRH^{559}
B. mori	N	Nucleocapsid	60.69	5.09	
	P	Phosphoprotein	36.51	6.05	^{275}RKPR^{280}
	M	Matrix protein	34.90	9.57	
	G	Glycoprotein	70.05	8.39	^{329}RHKR^{323}
	X	Unknown	**12.63**	5.42	
	L	Polymerase	24.42	8.23	^{9}RKKRP^{15}, ^{554}KKRH^{559}

[a]The molecular weights (MWs) of accessory proteins are in boldface font.
[b]NLS, nuclear localization signal.

addition to the vegetables tested in this study, *S. frugiperda* larvae generally feed upon members of the grass family, and the most common human-edible food plant in this category is sweet corn. Other human-edible food crops that are consumed by the larvae include apple, grape, orange, papaya, peach, and strawberry (http://entnemdept.ufl.edu/creatures/field/fall_armyworm.htm).

Characterization of Sf-RV proteins in plant cells. Sf-RV lacks a clearly identifiable movement protein, a key mark of plant-adapted viruses, suggesting that Sf-RV is unlikely to be adapted to replicate in plants. However, its common occurrence in

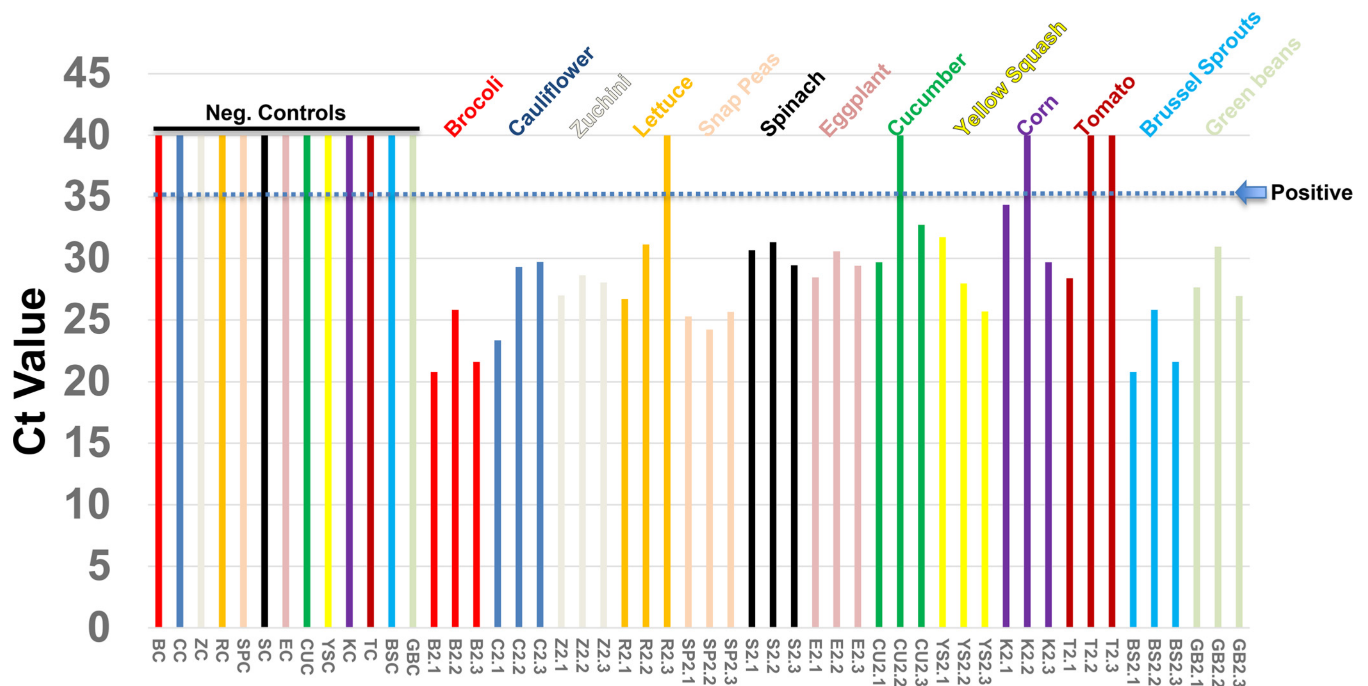

FIG 4 C_T values for detection of Sf-RV RNA on vegetables following overnight feeding by *S. frugiperda* larvae. C_T values >35 were considered negative for detection of Sf-RNA. Larvae, and their frass, were removed from the vegetables prior to RNA isolation.

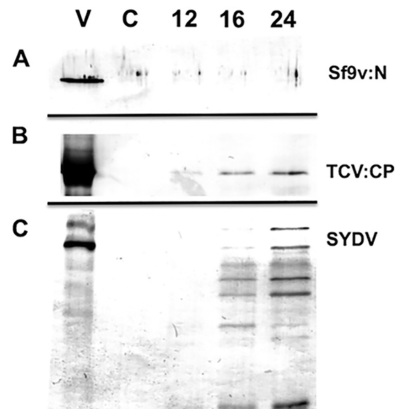

FIG 5 Immunodetection of viral proteins in a time course of equivalent amounts of *N. benthamiana* protoplasts inoculated with Sf-RV (A), *Turnip crinkle virus* (TCV) (B), or *Potato yellow dwarf virus* strain (SYDV) (C). Protoplasts were sampled at 12, 16, and 24 h after inoculation and probed via Western immunoblotting with polyclonal antibodies raised against the Sf-RV nucleocapsid (Sf-RV:N), TCV coat protein (TCV:CP), or disrupted SYDV virions, which detect a ladder of proteins corresponding to G, N, M, and P (SYDV). Purified virus (V) and uninoculated protoplasts (C) served as controls.

phytophagous insects and possible exposure to plants via feeding raised the possibility that plants could serve as reservoirs for Sf-RV and potentially amplify virus if replication was able to occur in initially infected cells. Moreover, we examined the characteristics of Sf-RV proteins in plants to determine whether their physicochemical properties might aid or inhibit adaption to plants. Protoplast experiments showed that under conditions that were conducive to the replication of a plant-adapted rhabdovirus *Potato yellow dwarf virus*, or plus-strand RNA *Turnip crinkle virus*, we were unable to detect replication of Sf-RV in protoplasts of *Arabidopsis thaliana* (data not shown) or *Nicotiana benthamiana*, plants that are known to be susceptible to a wide range of plant viruses (Fig. 5) (27). In *N. benthamiana*, results of Sf-RV protein localization in plant cells were consistent with that expected for rhabdoviruses, with the G and M proteins associated with membranes, particularly, perinuclear membranes. The short and long versions of the accessory protein were readily differentiated based on the ability to accumulate in nuclei. The phosphoprotein localized to the cell periphery, with no accumulation in nuclei (Fig. 6). Interestingly, the nucleocapsid protein accumulated at punctate loci on the cell periphery. These loci colocalized with the plasmodesma-associated protein PDLP1 (Fig. 7).

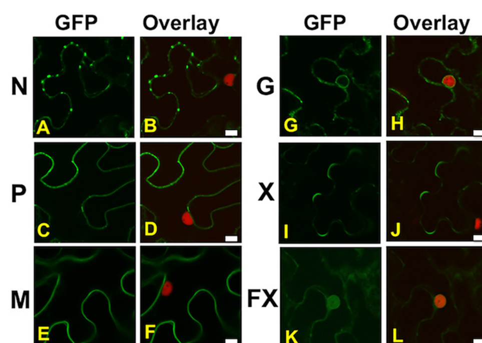

FIG 6 Localization of GFP:Sf-RV protein fusions in leaf epidermal cells of transgenic *N. benthamiana* expressing the nuclear marker fusion RFP:Histone2B by agroinfiltration. Localization patterns of GFP fusions are shown alone (GFP) or relative to the red nuclei of transgenic cells (overlay) for nucleocapsid protein (A and B), phosphoprotein (C and D), matrix protein (E and F), glycoprotein (G and H), the 9-kDa variant of the accessory protein (I and J), and the 13-kDa variant of the accessory protein (K and L). Scale bars, 10 μm.

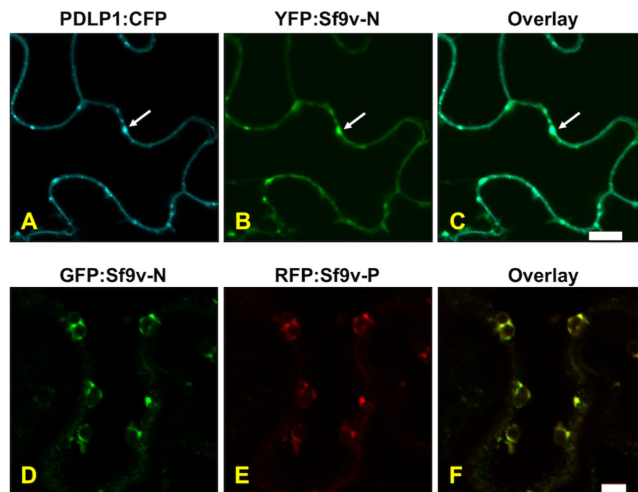

FIG 7 YFP:Sf-RV-N fusion has a localization pattern in leaf epidermal cells of *N. benthamiana* similar to that of the plasmodesmata marker fusion CFP:PDLP1. (A) CFP:PDLP1; (B) YFP:Sf-RV-N; (C) overlay of panels A and B. Scale bar, 10 μm. Coexpression of Sf-RV N and P proteins alters their expression patterns: GFP:Sf-RV-N (D), RFP:Sf-RV-P (E), overlay of panels D and E. Scale bar, 5 μm.

DISCUSSION

Virus discovery by next-generation sequencing has rapidly expanded our understanding of environs in the biosphere occupied by viruses. In many cases, these findings are surprising, as viruses are being identified in cell types that exhibit no overt cytopathic effects, as in the case of Sf9 rhabdovirus in Sf9 cell lines that have been in use for the production of vaccines and other biologicals used in humans. Cell lines cleared of Sf-RV are equivalent to virus-infected lines for the purposes of protein expression. However, established production facilities will continue to use Sf-RV-infected cell lines, which raises concerns about human exposure to this virus. In this study, we describe the first population-level studies that sought to determine how widespread Sf-RV is, and in which insect species it exists, in the wild.

Importantly, it is now well established that Sf-RV does not replicate in human cells, which is of paramount concern regarding human exposure. In contrast, our survey of *S. frugiperda* moths collected in hormone traps over a period of 5 years demonstrates that Sf-RV is endemic in wild populations in the southern United States. While broader surveys are warranted to gain a clearer perspective on the population structure of Sf-RV, it appears that there may be at least two lineages, based upon N gene sequences. It is expected that Sf-RV is transmitted vertically in insect populations; therefore, Sf-RV phylogenies should be congruent with the *S. frugiperda* population structure (4, 9, 28). Of significant interest is whether our findings for *S. frugiperda* may also be reflected in populations of *H. subflexa* and *B. mori*, cell lines which were found to be infected with Sf-RV. Given the plant host range of *S. frugiperda*, Sf-RV would have been predicted to be found in the similarly polyphagous *H. virescens* and not in *H. subflexa*, which feeds only on species in the genus *Physalis* (29). However, we found no evidence for Sf-RV replication in *H. virescens* cell lines, while the *H. subflexa* cell line tested was already infected with the virus. A cell line of *B. mori*, which also has a limited host range, was also determined to be infected with Sf-RV. A comparison of the genomes of Sf-RVs isolated from these three lepidopterans showed that the major differences lie in the sizes of the accessory proteins of unknown function. For reasons that are unclear, the accessory protein gene is unstable, and a 320-nucleotide portion is susceptible to deletion when infected cells lines are passaged in culture. In contrast, the 320-bp fragment was found to be present in all Sf-RV isolates from wild populations of *S. frugiperda*, suggesting that this gene is important in the context of insect infections. The full-length (12.63 kDa) accessory protein is capable of nuclear import, as shown in subcellular localization studies, whereas the truncated from (8.95 kDa) is excluded from

the nucleus. Many RNA viruses with cytoplasmic sites of replication encode proteins that target the nucleus to perturb gene expression in host cells (30–34). It is possible that such regulation is required for infection of insects. Alternatively, we cannot rule out that the presence of Sf-RV in *B. mori*- and *H. subflexa*-derived cell lines is due to contamination that may have occurred when these cells were cultured in labs working with Sf-9 cells. Examination of wild populations is therefore essential to resolve this issue.

Consistent with its broad host range, we found that Sf-RV RNA was deposited on a variety of vegetables by feeding *S. frugiperda* larvae. This finding raises the possibility that humans are exposed on occasion to this virus via consumption of food items that overlap the *S. frugiperda* host range. Although we found no detectable replication of Sf-RV in two species of plants, the localization patterns of proteins exhibit some peculiarities consistent with plant-adapted species (30). Most notable among these is the ability to target the cell periphery, particularly plasmodesmata, the cell junctions in plants that provide symplastic continuity between adjacent cells separated by otherwise impenetrable cell walls (35). Sf-RV lacks a clearly identifiable movement protein, which is a hallmark of plant-adapted viruses. However, the nucleocapsid protein of Sf-RV is clearly able to target plasmodesmata, suggesting that it could provide movement protein functions. Furthermore, as for the cognate proteins of their plant-infecting counterparts, coexpression of the N and P proteins results in the formation of a complex of both proteins in spherules at the cell periphery. While plant viruses vectored by lepidopterans are rare relative to those transmitted by aphids, planthoppers, and leafhoppers (36), it is of interest to know what additional factors contribute to the inability of rhabdoviruses in these insects to fully adapt to plant cells. In the case of Sf-RV, it appears that protein stability is a key factor. While we were able to localize proteins of this virus as fusions to full-length green fluorescence protein (GFP) or red fluorescent protein (RFP), it was impossible to detect interactions between these proteins using bimolecular fluorescence complementation under conditions now standard for generating plant rhabdovirus protein interaction and localization maps (37–40) (data not shown). Mechanical inoculation of plants, and protoplast transfection experiments, did not result in evidence for replication of Sf-RV in plants.

In conclusion, this report and other studies have demonstrated that Sf-RV does not replicate in plant cells (15–20). In addition, population, infectivity, and feeding studies described here demonstrate that Sf-RV is endemic in the environment with the capability for replication in up to four insect species, *S. frugiperda*, *S. exigua*, *H. subflexa*, and *B. mori*, each with the potential to deposit virus on human-consumed foods. Indeed, *S. frugiperda* larva feeding studies demonstrated that significant amounts of viral RNA were deposited on a variety of vegetables. Therefore, there is increasing evidence of a history of human exposure to Sf-RV, arguing that this virus is unlikely to pose a significant health hazard to humans.

MATERIALS AND METHODS

Sf-RV genome sequence analyses. The genomic sequence of Sf-RV found in the Takeda Vaccines Sf9 cell line was determined prior to the first published report of Sf-RV by Ma et al. (15). In experiments designed to screen for insect retroviruses, baculovirus lysates of Sf9 cells were clarified of debris and then subjected to Benzonase (Sigma-Aldrich) digestion to eliminate free (not particle-associated) nucleic acids. Following nuclease digestion, the material was centrifuged at $100{,}000 \times g$ through a 20% sucrose cushion in order to collect virus particles. High-speed pelleted material was then subjected to viral nucleic acid extraction and then treated with RNase-free DNase in order to remove baculovirus and insect genomic DNA, while leaving any viral RNAs intact. A random N9 primer tagged with a Not I restriction site at its 5′ end (TATTGCGGCCGCTTCTTNNNNNNNNN) was used for both the first-strand reverse transcriptase reaction and the second-strand Klenow DNA polymerase reaction, resulting in double-stranded DNAs that could be amplified using a PCR primer consisting of the Not I sequence tag (TATTGCGGCCGCTTCTT) and then cloned into a plasmid vector. Approximately 20% of the cloned fragments were shown to be distantly homologous to the L protein of rhabdoviruses following sequence analysis (DNA sequencing facility, UC Davis). The rhabdovirus homologies were observed at the amino acid sequence level only and were in the range of 24% to 26% homology through part of the L gene. Sequence analysis of all clones showing rhabdovirus homology as well as the clones that showed no detectable homology in the GenBank database resulted in the building of two large sequence contigs of approximately 7,000 and 4,500 nucleotides, respectively, with each containing a portion of an open

reading frame with homology to rhabdovirus L proteins. Suspecting that the two sequence contigs were part of the same viral genomic sequence, additional RT-PCR cloning using one primer from each contig resulted in the isolation of clones representing the sequences spanning these two contigs. Finally, rapid amplification of cDNA ends (RACE) techniques allowed for the isolation of 5′- and 3′-end clones, completing the sequence of the 13,267 nucleotide novel rhabdovirus genome. Additional RACE experiments performed at the University of Kentucky to define the 3′ and 5′ ends of the genome were performed using the BD-SMART RACE cDNA amplification kit according to the manufacturer's instructions (Thermo Scientific). For these analyses, cDNA was synthesized by Moloney murine leukemia virus (MMLV) reverse transcriptase, and PCRs were conducted with Advantage-II DNA polymerase (Clontech).

Sequence analysis of Sf-RV in HsAM1 and Bm-N cell lines. The *H. subflexa* cell line HsAM1 (gift from Cindy Goodman, USDA, ARS, Midwest Area Biological Control of Insect Research Laboratory, Columbia, MO) and the *B. mori* cell line BM-N. (ATCC) were found to be infected with Sf-RV by using PCR analyses. Sf-RV genome sequence analyses in these cell lines were accomplished by reverse transcriptase PCR amplification of overlapping fragments from cellular RNA followed by direct sequencing of purified PCR fragments (DNA sequencing facility, UC Davis). PCR primers were based on the known Sf9 virus genome sequence as determined by Takeda and also published by Ma et al. (15). Sequence data from the *H. subflexa*-derived fragments and the *B. mori*-derived fragments were analyzed by the ContigExpress program of the Vector NTI suite in order to assemble the individual fragment sequences into contigs.

Quantification of Sf-RV RNA by PCR. A TaqMan quantitative RT-PCR (qRT-PCR) assay was developed in order to quantify Sf9 rhabdovirus minus-strand RNA. The primer and probe sequences are located in a 64-nucleotide target sequence within the L gene. The primer and probe sequences are as follows: forward (FWD), TCTGTATTATGGGTTTGATCAGCTAAG; reverse (REV), CTCGCTGCTGAGCGGTTT; probe, 6-carboxyfluorescein (6FAM)-AGGATTGGAGAATTATAC.

A standard curve RNA for quantification purposes was prepared by *in vitro* transcription of a plasmid clone containing the L gene target sequence using T7 RNA polymerase following linearization of the plasmid DNA. Template plasmid DNA was subsequently digested with DNase, and the standard curve RNA was purified (RNeasy; Qiagen). An RNA of approximately 5 kb was expected, but two RNAs of equal intensities, 3.5 kb and 5 kb, were produced as shown by glyoxal-agarose gel analysis. The smaller of the two RNAs is large enough to contain the target sequence and was likely produced as a result of premature transcription termination. For quantification purposes, the average molecular weight of the standard curve RNA was therefore 4.25 kb or 1.4×10^6 Da. Use of this RNA as a standard curve in the TaqMan assay demonstrated a reproducible limit of detection of 0.01 fg of RNA, which is equivalent to 4 molecules of standard curve RNA. Sf-RV RNA quantification was performed using the TaqMan RNA-to-Ct 1 Step kit (Thermo Fisher) and the ABI 7500 real-time PCR system. Alternatively, PCR quantification was achieved without the use of an RNA standard curve by employing the RainDrop digital PCR system (RainDance Technologies). For PCR assays of RNA derived from *S. frugiperda* moth or larvae specimens in which quantification of RNA copy number was not performed, cycle threshold values were reported from experiments using the L gene primer and probe set and the ABI 7500 real-time PCR system. An additional primer and probe set contained within the common 320-nucleotide accessory gene deletion region was also employed.

Sf-RV replication studies in insect cell lines. The following insect cell lines were obtained from Cindy Goodman (USDA, ARS, Midwest Area, Biological Control of Insect Research Laboratory, Columbia, MO) (cell line name/species and tissue of origin): BCIRL/AMCY-SeE1/*Spodoptera exigua* eggs, BCIRL/AMCY-SeE4/*S. exigua* eggs, BCIRL/AMCY-SeE5/*S. exigua* eggs, *Heliothis virescens* ovaries, BCIRL/HvAM1/*Heliothis virescens* ovaries, BCIRL/HzAM1/*Helicoverpa zea* ovaries, BCIRL-HzFB33/*H. zea* fat bodies, BCIRL-HsAM1/*Heliothis subflexa* ovaries, and BCIRL-AgAM1/*Anticarsia gemmatalis* ovaries.

To determine the potential for Sf-RV to replicate in these cell lines, a T25 tissue culture flask of each cell line containing 10 ml of EX-Cell 420 medium plus 10% fetal bovine serum (FBS) was exposed to the Sf9 rhabdovirus by removing 5 ml of the culture medium and replacing it with 5 ml of conditioned medium from Sf9 cells that had been centrifuged and filtered through a 0.2-μm membrane in order to ensure no Sf9 cell carryover. The Sf9 conditioned medium was allowed to remain on the cell cultures for 24 h, after which the medium was removed and the insect cell lines were further cultured in EX-Cell 420 medium containing 10% FBS. Upon reaching confluence, approximately 10% of the cells of each culture were collected and stored as a frozen cell pellet (p0), while new subcultures of each were established for continued growth and passaging. In this way, two additional frozen cell pellets of each cell line were collected for passage numbers p1 and p2, respectively.

Following collection of the frozen cell samples, total cellular RNA was purified from each cell sample (RNeasy; Qiagen) as well as from control cells from each cell line that were not exposed to the Sf9 rhabdovirus (preinfected). Each RNA sample was tested in the Sf9 rhabdovirus qRT-PCR assay described above for detection and quantification of any rhabdovirus RNA signals. Data were expressed in terms of femtogram rhabdovirus RNA per microgram of total cellular RNA.

Two additional Sf-RV replication experiments were performed in *S. exigua* cells in which the Sf9 cell-conditioned medium was serially diluted to reach the endpoint of infectivity in order to determine the number of infectious units per total number of Sf-RV genomic RNA copies. Using the Spearman-Karber method of 50% tissue culture infective dose ($TCID_{50}$), the calculations for both virus preparations showed a similar rhabdovirus titer of 2×10^3 infectious units per ml (22). Based on the original rhabdovirus particle count determined by qRT-PCR, the virus for which the titer was determined in experiment 602-132 had an infectious-to-noninfectious ratio of 1:17,000, whereas the virus for which the titer was determined in experiment 602-170 had an infectious-to-noninfectious ratio of 1:26,000.

Measurement of Sf-RV in adult *S. frugiperda* specimens collected from the environment. RNA samples isolated from the collected moth specimens were analyzed for the presence of Sf9 rhabdovirus RNA by 2 different TaqMan RT-PCR assays (see above) specific for a target sequence in the L (polymerase) gene and a second target sequence in the accessory gene within the boundaries of the common 320-nucleotide deletion. The accessory gene target sequence is in an unstable area of the Sf9 rhabdovirus genome in a 320-nucleotide (nt) region that is sometimes deleted. Negative-control samples produced a C_T value of ≥40, consistent with the absence of contaminating rhabdovirus sequences producing false-positive results.

Insect feeding assay. *S. frugiperda* eggs were acquired from Benzon Research, Inc. (Carlisle, PA, USA) and were hatched under optimal controlled environmental conditions in the presence of an artificial lepidopteran diet acquired from the insect supplier. At approximately day 14 posthatching and slightly before the insects were to begin pupating, the larvae were placed on a variety of vegetables and allowed to feed for 18 h (overnight). The vegetables included broccoli, cauliflower, zucchini, romaine, snap peas, spinach, eggplant, cucumber, yellow squash, corn, tomato, Brussels sprouts, and green beans. After overnight feeding, samples of the vegetables where insect feeding had taken place were excised and subjected to RNA extraction and RT-PCR analysis to assay for the presence of Sf-RV viral RNA.

Protoplasts and immunoblotting. Protoplasts were isolated from 1 g of *N. benthamiana* leaves from young plants at the 4- to 6-leaf stage of growth, as described by Panaviene et al. (41). Experiments were always conducted in quadruplets, with each lot of protoplasts, in a final suspension of 1 ml, being split into 250-μl aliquots. One aliquot served as the uninoculated control, while the remaining three were inoculated with Sf-RV (using 5.5×10^7 particles), while the other two were inoculated with *Potato yellow dwarf virus* (PYDV) or *Turnip crinkle virus* (TCV) by the addition of 100 μl of clarified supernatant after grinding 0.1 g of infected leaf tissue in 1 ml of protoplast isolation buffer. At 12, 16, and 24 h postinoculation, 50 μl of protoplast suspension was sampled, mixed with equal volumes of SDS-PAGE loading buffer, and processed for Western immunoblotting, essentially as described previously (42).

Protein localization in plant cells. To our knowledge, the subcellular localization patterns of Sf9-RV proteins have not been determined. Heterologous expression systems are generally valid for localization studies given the conservation of subcellular localization signals (i.e., the same nuclear localization signal functions correctly if expressed in yeast, plant, or mammalian cells) (43–45). As such, the plant-based system used is appropriate for these studies (39, 46). One of the aims of this study was to determine the replication competency of Sf9-RV in plants given the opportunity for transmission by its phytophagous host. We chose to use *N. benthamiana* for protein localization studies, which is widely used for such experiments (27, 43). Furthermore, initial phylogenetic comparisons suggested that Sf9-RV is related to lettuce necrotic yellows virus, a plant-adapted rhabdovirus (data not shown). Therefore, plant-based expression was necessary to determine if Sf9-RV encoded a protein that could target the virus to the cell periphery, in lieu of a clearly identifiable movement of protein. To accomplish the expression of GFP protein fusions in plant cells, full-length ORFs of Sf-RV were amplified by PCR, cloned, and sequenced verified. These clones were used to express fusions to fluorescent proteins in leaf epidermal cells, essentially as described previously for the construction of rhabdovirus protein interaction and localization maps (30, 39, 46, 47).

Accession number(s). The Sf-RV genome sequences from *B. mori*, *H. subflexa*, and Sf9 cells (Takeda cell culture), were deposited in GenBank as accessions MH926029, MH926030, and MH926031.

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