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Narinda Pal
Iowa State University

Sandhya Boyapalle
Iowa State University

Randy Beckett
Iowa State University

W. Allen Miller
Iowa State University, wamiller@iastate.edu

Bryony C. Bonning
Iowa State University, bbonning@iastate.edu

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Abstract

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Comments

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A Baculovirus-Expressed Dicistrovirus That Is Infectious to Aphids[▽]

Narinder Pal,[1] Sandhya Boyapalle,[1]† Randy Beckett,[2] W. Allen Miller,[2] and Bryony C. Bonning[1]*

Departments of Entomology[1] and Plant Pathology,[2] Iowa State University, Ames, Iowa 50011

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Detailed investigation of virus replication is facilitated by the construction of a full-length infectious clone of the viral genome. To date, this has not been achieved for members of the family *Dicistroviridae*. Here we demonstrate the construction of a baculovirus that expresses a dicistrovirus that is infectious in its natural host. We inserted a full-length cDNA clone of the genomic RNA of the dicistrovirus *Rhopalosiphum padi virus* (RhPV) into a baculovirus expression vector. Virus particles containing RhPV RNA accumulated in the nuclei of baculovirus-infected Sf21 cells expressing the recombinant RhPV clone. These virus particles were infectious in *R. padi*, a ubiquitous aphid vector of major cereal viruses. The recombinant virus was transmitted efficiently between aphids, despite the presence of 119 and 210 vector-derived bases that were stably maintained at the 5′ and 3′ ends, respectively, of the RhPV genome. The maintenance of such a nonviral sequence was surprising considering that most RNA viruses tolerate few nonviral bases beyond their natural termini. The use of a baculovirus to express a small RNA virus opens avenues for investigating replication of dicistroviruses and may allow large-scale production of these viruses for use as biopesticides.

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Small RNA viruses (SRVs) (<60 nm in diameter) are common in insect populations and are known to induce epizootics (13), but they have received little attention for use as biopesticides. This may be due to the perceived difficulty of production, either in vivo because of a lack of product purity or in vitro because of the lack of cell lines that support replication (13). In contrast, considerable attention has been paid to the large-scale production of baculoviruses both for use as protein expression vectors and for use as biopesticides (4, 23). Although the baculovirus expression system has been used for the production of a wide range of virus particles (9), baculovirus expression of a heterologous infectious virus is rare (21, 28). We know of no examples where a baculovirus-expressed virus has been demonstrated to infect its natural host. Here we describe the construction of a full-length clone of the RNA genome of *Rhopalosiphum padi virus* (RhPV) and baculovirus-mediated expression of the clone to produce virions that are infectious to aphids. This study suggests that baculovirus vectors may be useful for the production of SRV biopesticides (11, 12, 30, 42).

RhPV was first isolated from the bird cherry-oat aphid, *R. padi*, a pest of members of the family *Gramineae*, especially maize, barley, oats, and wheat. *R. padi* is the major vector of many cereal viruses such as *Barley yellow dwarf virus* (38). Both *R. padi* and *Barley yellow dwarf virus* occur worldwide, wherever cereals are grown. RhPV infection decreases aphid longevity and fitness and reduces aphid colony populations (15). RhPV circulates within the phloem of plants, thereby using plants as passive reservoirs for transmission (20). RhPV belongs to the picornavirus-like *Dicistroviridae* family and has a positive-sense RNA genome of 10,011 nucleotides (nt) (31) with a characteristic dicistronic genome organization (Fig. 1) (14).

The study of viruses has benefited greatly from the ability to engineer specific mutations into the viral genome (35), and the production of a cDNA clone from which infectious RNA can be transcribed is an important step in the development of reverse genetic systems for all positive-strand RNA viruses. Thus, our goal was to construct an infectious cDNA clone of the RhPV genome to gain insight into the control of gene expression, replication, assembly, and structure of a replicating dicistrovirus. The genomes of several dicistroviruses have been sequenced (22, 25, 41, 44). Dicistroviruses harbor two very different, well-characterized internal ribosome entry sites (IRESs) that facilitate the translation of the two open reading frames ORFs (36, 40). The structure of the cricket paralysis dicistrovirus virion is known at high resolution (43). Production of infectious transcripts from a PCR-derived template of black queen cell virus has been reported (3); however, there have been no reports of an infectious clone of a dicistrovirus. Here we report the construction of a clone of the RhPV genome that is expressed from a baculovirus vector and which tolerates nonviral terminal bases while being infectious in aphids for several generations. This clone opens the way to the study of RhPV RNA replication.

MATERIALS AND METHODS

Virus and cells. *Spodoptera frugiperda* cells (line IPLB-Sf21) (45) and Sf9 cells were maintained as described previously (5). A *polh*-negative recombinant baculovirus expressing bacterial β-galactosidase, AcRP23lacZ, was used as a control virus (37).

Construction of a full-length cDNA clone of RhPV. The recombinant baculovirus AcRhPV, which contains the cDNA of RhPV under the control of the *polh* promoter, was constructed with the Bac-to-Bac baculovirus expression system (Gibco-BRL/Invitrogen).

RhPV virions were purified from infected *R. padi* by sucrose gradient centrifugation (16), and RhPV RNA was extracted from virions by using the Absolutely RNA reverse transcription (RT)-PCR miniprep kit (Stratagene, La Jolla, CA). For construction of RhPV cDNA fragments (Fig. 1), first-strand cDNA was synthesized from 1 μg viral RNA by Moloney murine leukemia virus H reverse transcriptase (Gibco-BRL/Invitrogen) at 42°C for 1 h. The GeneAMP XL PCR

* Corresponding author. Mailing address: Department of Entomology, 418 Science II, Iowa State University, Ames, IA 50011-3222. Phone: (515) 294-1989. Fax: (515) 294-5957. E-mail: bbonning@iastate.edu.

† Present address: H. Lee Moffitt Cancer Center & Research Institute, University of South Florida, Tampa, FL 33612.

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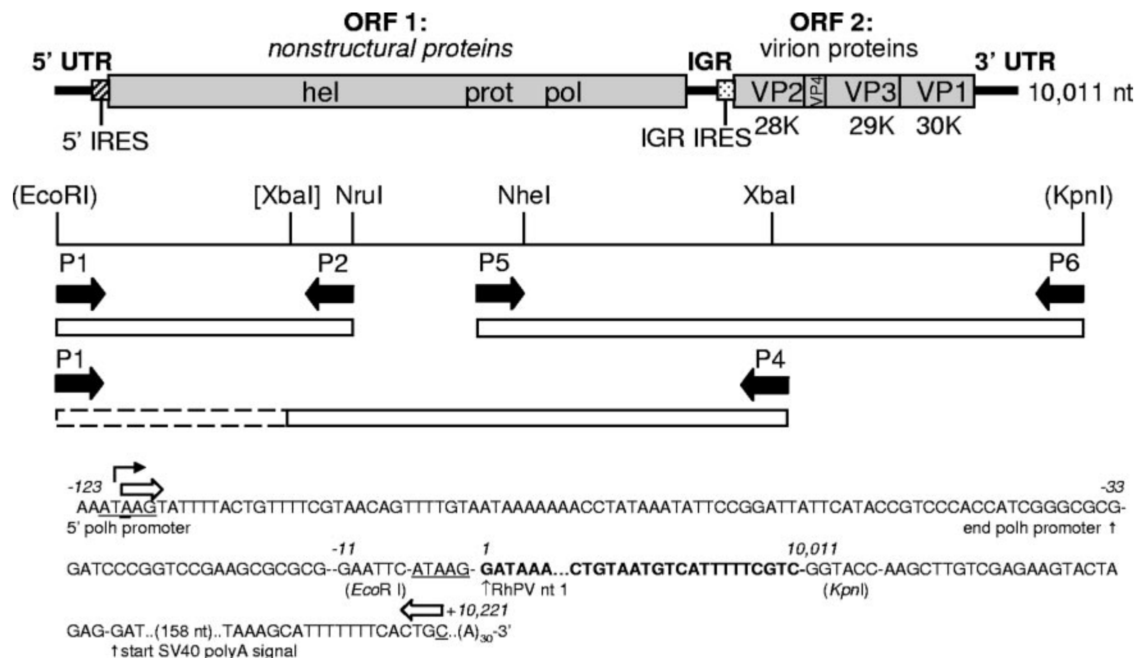

FIG. 1. Cloning strategy, map of the full-length RhPV cDNA clone in the baculovirus expression vector pFastBac1, and sequences of genome termini. (Top) Genome organization of RhPV RNA. UTR, untranslated region; IGR, intergenic region; hel, helicase domain; prot, proteinase domain; pol, RNA-dependent RNA polymerase active site; VP, virion protein. The positions of the PCR primers (P1 to P6) used for the amplification of cDNA clones in relation to the RhPV RNA genome are indicated by the solid arrows below the primer designations and above the amplified cDNA fragments (open rectangles). EcoRI and KpnI restriction sites were introduced artificially (indicated by parentheses) into the PCR primers at the 5′ and 3′ ends, respectively. The three cDNA fragments were sequentially introduced into pFastBac1 so that the viral genome rests between the EcoRI and KpnI sites (positions −11 and 10,011, respectively), as described in Materials and Methods. The sequence below the map shows the RhPV sequence insert in bold and the flanking vector sequence, which includes the *polh* core promoter sequences (underlined) and SV40 polyadenylation/termination signal (bottom line). The open arrows and singly underlined bases indicate the 5′ and 3′ ends detected by RACE following the passaging of baculovirus-derived virions in aphids for 32 days.

kit (PE Applied Biosystems, Foster City, CA) was also used in accordance with the manufacturer's specifications. To generate the first cDNA fragment (leftmost open rectangle in Fig. 1), we used primer P2 (nt 3224) (5′-TCGCGAACAATA GATAGGCACT-3′ [the NruI site is underlined]) for first-strand synthesis and primer P1 (5′-GGGGGAATTC*ATAAG*GATAAAAGAACCTATAATCCCTT CGCA-3′ [the introduced EcoRI site is underlined; the introduced *polh* core promoter sequence, ATAAG, is italicized]). The resulting RT-PCR product was cloned between the EcoRI and StuI sites in the multiple cloning site of the pFastBac1 baculovirus expression vector and named pclone 1. We attempted to produce the second fragment from nt 2149 (EcoNI) to nt 7070 (XbaI) but found that pclone 1 had an unexpected XbaI site at approximately nt 2000 (in square brackets, Fig. 1), which prevented cloning with XbaI. Hence, we used P1 and P4 (nt 7070) (5′-CTCTCCTGTGACGTATCTAGAC-3′ [the XbaI site is underlined]) to generate a 7-kb PCR product that was cut with XbaI. The larger (~5-kb) fragment was gel purified with the QIAquick gel purification kit (QIAGEN, Valencia, CA). pclone 1 was also cut with XbaI, and the larger fragment (~6,775 bp), which included pFastBac1 (4,775 bp) and a 2-kb 5′-end fragment of the RhPV sequence, was gel purified. The two gel-purified products were ligated and transformed into competent *Escherichia coli* DH5α (Promega, Madison, WI), and the resulting clones were named pclone 2. The third cDNA fragment (rightmost open rectangle in Fig. 1) was generated with P5 (nt 5680; 5′-CCGCACG TTATAGCTGGCGACTATT-3′) (upstream of the NheI site at nt 6522) and P6 (nt 10011; 5′-GGGGGGTACCGACGAAAAATGACATTACAG-3′ [the introduced KpnI site is underlined]). The amplified product was cloned between the NheI and KpnI sites of pclone 2, thus generating full-length cDNA clones of the RhPV genome minus the poly(A) tail. The resulting recombinant baculovirus transfer vectors were named pAcRhPV1 to -12. Four full-length clones selected on the basis of restriction enzyme analysis were sequenced across the cloning junctions to confirm their sequences.

Generation of recombinant baculoviruses. The *polh*-negative recombinant baculoviruses AcRhPV6, AcRhPV7, AcRhPV11, and AcRhPV12 were generated according to the manufacturer's protocols. Transfection experiments were carried out with Sf9 cells and Lipofectin (Invitrogen). Seven days posttransfection, cell supernatants were harvested and recombinant viruses were isolated by two rounds of plaque purification with Sf21 cells. Individual plaque isolates were later amplified following the confirmation of RhPV expression by immunoblot analysis of the infected-cell lysates with purified polyclonal RhPV antiserum (5). The titers of virus stocks were determined by plaque assay by standard procedures (34). AcRhPV6 was used in further experiments.

Infection of Sf21 cells. Sf21 cells (3×10^6 cells in a 25-cm^2 flask) were infected with recombinant AcRhPV6 or the control virus AcRP23lacZ at a multiplicity of infection (MOI) of 5 or 10. After 1 h at room temperature, the virus was removed and 5 ml medium was added to the flasks. Cells were incubated at 28°C for 4 to 5 days and harvested for further analysis.

Purification of virus particles. Virus particles were purified from Sf21 cells infected with recombinant AcRhPV6 or AcRP23lacZ at 5 days postinfection (dpi) by sucrose gradient centrifugation as described by Krishna et al. (28). Purified virus particles were negatively stained with 2% uranyl acetate and examined with a JEOL 1200EX scanning/transmission electron microscope (STEM) at 80 kV.

Immunogold electron microscopy. Sf21 cells (3×10^6 cells per 25-cm^2 flask) were infected with AcRhPV6 or AcRP23lacZ at an MOI of 10, and the cells were harvested 5 dpi. The cells were pelleted and processed as described previously for examination with a JEOL 1200EX STEM at 80 kV (5).

Detection of RhPV RNA. For Northern blot assays, total cellular RNA was purified from AcRhPV6-infected Sf21 cells at 4 and 5 dpi and from AcRP23lacZ-infected cells at 5 dpi with TRIzol (Invitrogen) according to the manufacturer's specifications. RNA was extracted from purified virus particles with the Absolutely RNA RT-PCR miniprep kit (Stratagene). The RNA concentration was determined by measurement of absorbance at 260 nm. RNA was analyzed by Northern blot assay as described by Koev et al. (27). Approximately 100 ng of RhPV RNA was run as a positive control. A ^{32}P-labeled RNA probe complementary to the 1.9-kb 3′-terminal sequence of RhPV was used to detect positive-sense RNA in the cells.

To detect positive- and negative-strand RhPV RNAs in AcRhPV6-infected Sf21 cells and aphids, RT-PCR was used with the primers pRhPV7 (5′-CAGA

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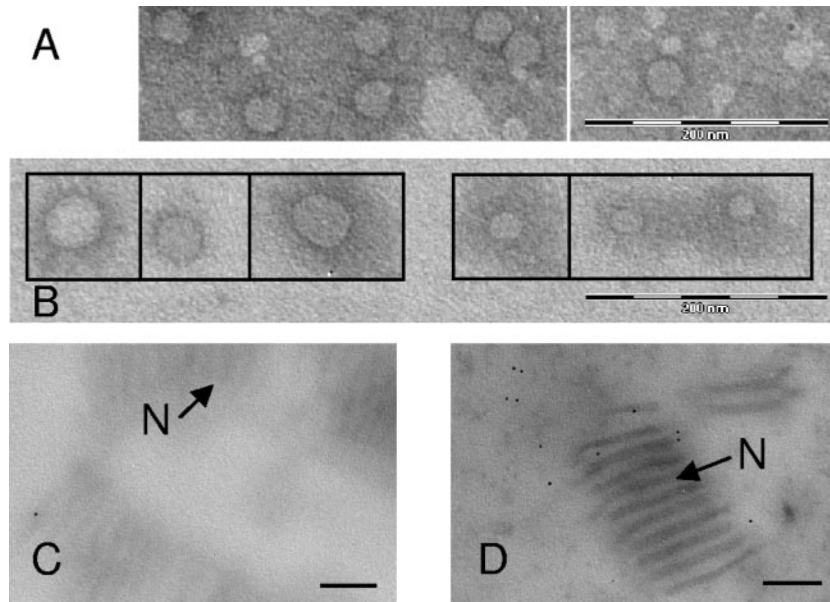

FIG. 2. Accumulation of RhPV particles and proteins in baculovirus-infected cells. (A) Electron micrograph of negatively stained *R. padi*-derived virus particles of wild-type RhPV. (B) RhPV particles recovered from Sf21 cells infected with AcRhPV6. Images of RhPV particles (at right) and larger particles of unknown identity (at left) from the same micrograph are shown for direct comparison. (C and D) Immunogold labeling of baculovirus-produced RhPV proteins. Sf21 cell infected with AcRP23LacZ (C) or AcRhPV6 (D). RhPV-specific antibody binding in the nucleus was detected by goat anti-rabbit serum conjugated with 10-nm colloidal gold. Baculovirus nucleocapsids (N), which accumulate in the nucleus prior to occlusion, are indicated. All bars represent 200 nm.

ACTAGGCCTTCAGCTT-3′; nt 6449) and P6 (nt 10011), resulting in a 3,562-bp RT-PCR product.

Aphid infectivity assays. A virus-free colony of *R. padi* was established and maintained in a separate building from the RhPV-positive colony. The virus-free colony was tested repeatedly for the presence of virus by Western blot assay and RT-PCR (5). For acquisition of virus by membrane feeding (10, 39), aphids were allowed to feed for 16 h on purified virus preparations derived from two 75-cm^2 flasks that had been infected with AcRhPV6 or AcRP23lacZ (MOI of 10) and harvested at 5 dpi. Infected-cell pellets were resuspended in 50 μl 0.01 M phosphate buffer (pH 7.0). The virus suspension was mixed with 50% sucrose in 0.01 M phosphate buffer, pH 7 (25% final concentration of sucrose). Control aphids were fed on 25% sucrose in 0.01 M phosphate buffer, pH 7. After the virus acquisition period, aphids were transferred to separate cages and maintained on oats. Three replicates were prepared with at least 30 aphids per replicate. Aphids were tested for RhPV infection from 2 weeks after virus acquisition by RT-PCR and Western blot assay. Total RNA was extracted from at least 10 aphids with TRIzol. First-strand synthesis was conducted by using the Superscript First Strand Synthesis kit (Invitrogen). RT-PCR was conducted with primers pRhPV7 and P6 (described above) by using 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 4 min, followed by a final extension at 72°C for 10 min. Aphids were also tested for virus infection by Western blot analysis and transmission electron microscopy.

Virus purification from aphids for Western blot analysis and transmission electron microscopy. Virus was purified from at least 20 aphids from each treatment (aphids fed with baculovirus-derived virus particles, virus-free aphids, and RhPV-positive aphids) as described previously (10, 39). Western blot analysis was conducted for detection of RhPV proteins as described previously (5). A purified virus suspension (10 μl) was negatively stained with 1% phosphotungstic acid (pH 6.2) for 30 s, placed on a Formvar-coated nickel grid, and examined under a JEOL 1200 EX STEM at 120 kV.

Aphid transmission of RhPV6. To test for aphid-to-aphid transmission of RhPV6, eight aphids were isolated on separate plants 2 weeks after aphid acquisition of baculovirus-derived virus particles. These aphids produced 30 to 40 progeny over the course of 17 days. Five of these progeny aphids per plant were tested for the presence of RhPV by RT-PCR. The primers used were RPF841 at nt 848 (5′-TTAATTTCGAACCCCGTCAG-3′) and RPR2081 at nt 2386 (5′-C TCAGTTTCGGGCTCTCTTG-3′). The PCR conditions used were 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 10 min. To confirm that the RT-PCR products obtained resulted from baculovirus-derived RhPV6 and not from contaminating wild-type RhPV, RT-PCR was used to amplify the sequence of the 5′ end of the RhPV6 RNA in aphids fed with baculovirus-derived virus particles and in AcRhPV6-infected Sf21 cells. The primers used were RhPVEcoR1 (5′-GCGCGGAATTC*ATAAG*GATA-3′) at −16 nt (the introduced EcoRI restriction site is underlined, and the *polh* core promoter sequence is italicized) and RPR2081 at nt 2386 (5′-CTCAGTTTCGGGCTCTCTTG-3′). The PCR conditions used were 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 10 min.

5′- and 3′-end sequences of RhPV6 RNA. To determine the sequences at the 5′ and 3′ ends, the SMART RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech, Mountain View, CA) was used according to the manufacturer's protocol. Total RNA was extracted with TRIzol (as described above) from aphids fed with baculovirus-derived virus particles and from baculovirus-infected Sf21 cells. One microgram of total RNA was then used for the preparation of 5′ and 3′ RACE-ready cDNA with the 5′ and 3′ RACE CDS primers, respectively. The first-strand synthesis primer for 3′ RACE included poly(T) for RT of RNA with poly(A) tails. RACE PCRs were then performed with the nested universal primer provided in the kit along with pRhPV4 at nt 2249 (5′-CGCACCACTCATATTGGCTTCAGTC-3′) for the 5′ RACE PCR or pRhPV7 at nt 6449 (5′-CAGAACTAGGCCTTCAGCTT-3′) for the 3′ RACE PCR. The PCR products were then excised from the gel, purified with the QIAGEN gel extraction kit (QIAGEN Inc., Valencia, CA), and either sequenced directly or cloned into pCR Blunt with the Zero Blunt PCR cloning kit (Invitrogen, Carlsbad, CA) prior to sequencing.

RESULTS

Baculovirus expression of RhPV6. A full-length cDNA clone of RhPV, called RhPV6, was assembled by ligating three overlapping RT-PCR products (Fig. 1) and cloned into the baculovirus vector pFastBac1. Recombinant baculovirus AcRhPV6 was then constructed for expression of the RhPV6 genome with the Bac-to-Bac expression system. AcRhPV6 contains an authentic polyhedrin promoter (*polh*), located 121 nt upstream of the 5′ end of the RhPV genome sequence, and the core

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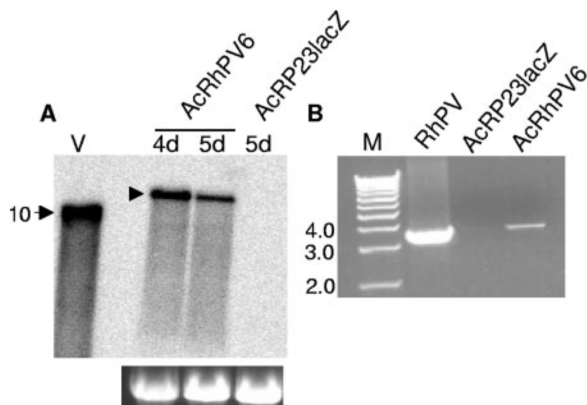

FIG. 3. Detection of RhPV RNA in (A) recombinant baculovirus infected Sf21 cells and (B) baculovirus-produced virus particles. (A) Northern blot hybridization of total cellular RNA from Sf21 cells infected with AcRhPV6 at 4 and 5 dpi or AcRP23lacZ at 5 dpi. Lane V contains 100 ng of viral RNA (10 kb). ^{32}P-labeled RNA complementary to the 3′ end of the RhPV genome was used as a probe. The bottom panel represents ethidium bromide-stained rRNA (used as a loading control). (B) RT-PCR of RNA extracted from purified virus particles. Lane M, 1-kb double-stranded DNA ladder; RhPV, RhPV RNA from aphid-derived virions; AcRP23lacZ, RNA extracted from AcRP23LacZ-infected Sf21 cells; AcRhPV6, RNA extracted from purified RhPV particles from AcRhPV6-infected Sf21 cells.

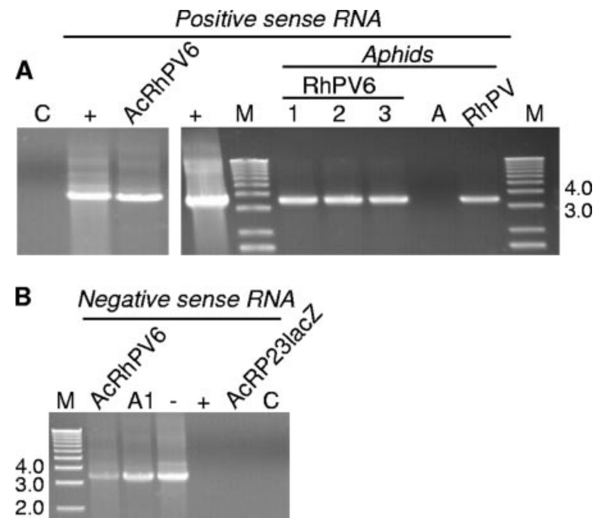

FIG. 4. Detection of (A) positive- and (B) negative-sense RhPV RNAs in AcRhPV6-infected Sf21 cells and in aphids fed on baculovirus-derived virus particles by RT-PCR. Lane M, 1-kb double-stranded DNA ladder. (A) Templates: C, control with no reverse transcriptase; +, positive-sense T7 in vitro transcript of RhPV RNA; AcRhPV6, AcRhPV6-infected Sf21 cells; 1 through 3, total RNA from three replicates of aphids 17 days after feeding on baculovirus-derived RhPV6 virus particles; A, virus-free aphids; RhPV, RhPV-infected aphids. (B) Templates: AcRhPV6, total RNA from AcRhPV6-infected Sf21 cells; A1, total RNA from aphids fed on baculovirus-derived RhPV6 virus particles; −, positive control, negative-sense transcript (SP6); +, negative control, positive-sense transcript (T7); AcRP23 lacZ, RNA from AcRP23lacZ-infected Sf21 cells; C, control with no reverse transcriptase.

baculovirus late promoter (ATAAG), immediately upstream of the RhPV genome (Fig. 1). The simian virus 40 (SV40)-derived transcription termination/polyadenylation signal extends 210 bp downstream of the 3′ end of the RhPV genome [not counting the poly(A) tail]. Thus, we expected the transcript from this construct to be significantly longer than the RhPV genomic RNA.

Infection of Sf21 cells with AcRhPV6, but not with the control virus AcRP23lacZ, yielded isometric virus particles similar to RhPV virions (Fig. 2A and B). Larger particles 45 to 47 nm in diameter were also detected in the infected-cell lysates (Fig. 2B). Baculovirus nucleocapsids, which would be deposited with their long axis parallel to the grid, were separated from RhPV particles by sucrose density gradient purification and hence not seen in purified virus preparations. RhPV virion proteins were detected by immunogold labeling of sections of infected Sf21 cells with purified anti-RhPV antibody. Labeling was observed in the nuclei of cells infected with AcRhPV6 (Fig. 2D). There was no labeling in the cytoplasm of AcRhPV6-infected cells or anywhere in cells infected with AcRP23lacZ (Fig. 2C).

Production and packaging of RhPV6 RNA. To determine whether recombinant baculovirus expression produced RhPV RNA in insect cells, Sf21 cells were infected with AcRhPV6 or AcRP23lacZ at an MOI of 10 and harvested at 4 or 5 dpi. Northern blot hybridization with an RhPV-specific probe detected RhPV RNA in Sf21 cells infected with AcRhPV6 at 4 and 5 dpi (Fig. 3A) and not in cells infected with negative control AcRP23LacZ. As expected, the baculovirus-derived RhPV RNA was significantly larger than the 10-kb wild-type RhPV RNA positive control (Fig. 3A).

To determine whether RhPV RNA was packaged into the virus particles, RNA was extracted from virus particles purified from infected Sf21 cells at 5 dpi and analyzed for the presence of RhPV RNA by RT-PCR (Fig. 3B). RT-PCR products of the expected size (3.6 kb) were acquired from virus particle-derived RNA and from the positive control viral RNA (Fig. 3B), confirming that RhPV RNA was packaged into baculovirus-produced virus particles. With the same primer pair (P6 and P7; see Materials and Methods), both positive- and negative-sense RhPV RNAs were also detected in AcRhPV6-infected Sf21 cells by RT-PCR (Fig. 4). To detect positive-sense RNA, negative-sense primer P6 was used for first-strand cDNA synthesis, while to detect negative-sense RNA, positive-sense primer P7 was used for first-strand cDNA synthesis. The presence of negative-sense RhPV RNA indicates the replication of RhPV RNA in Sf21 cells.

RhPV6 infectivity in aphids. We next investigated whether the virus particles generated by AcRhPV6 could infect aphids. Baculovirus-produced virus particles were purified by sucrose density gradient sedimentation and fed through Parafilm membranes to approximately 30 virus-free aphids (*R. padi*) for 16 h. The aphids were then placed on virus-free plants. Aphids were positive for the presence of both positive- and negative-sense RhPV RNAs 17 days postacquisition, as determined by RT-PCR in three of three replicates (Fig. 4). No PCR products were obtained from the negative control, uninfected aphids. RT-PCR performed with a 5′ primer (RhPVEcoRI) composed primarily of vector-derived, non-RhPV sequence predicted to be upstream of the 5′ end of the wild-type RhPV genomic sequence confirmed that infection of aphids resulted from

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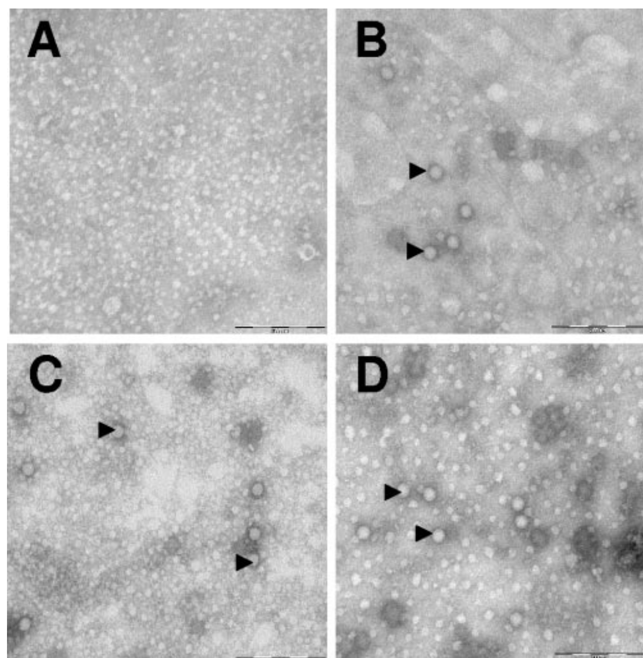

FIG. 5. Transmission electron micrographs of RhPV6 particles derived from aphids. A, virus-free aphids (negative control); B, RhPV-infected aphids (positive control); C and D, baculovirus-derived RhPV6 virus particles obtained 47 dpi of aphids. Representative stained virus particles approximately 27 nm in diameter in the virus minipreps are indicated by arrowheads. All scale bars represent 200 nm.

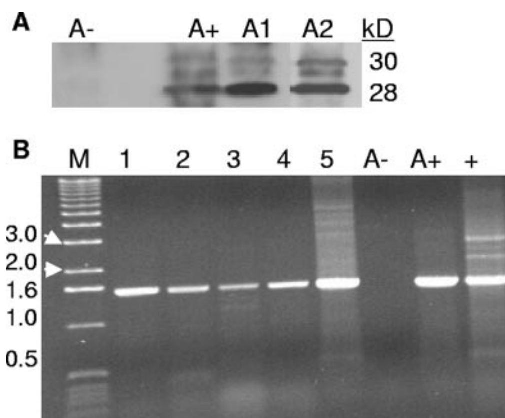

FIG. 6. Stability and transmission of RhPV in aphids. (A) Western immunoblot analysis of virus preparations obtained from aphids at 47 dpi with antibody against RhPV virions. Protein was extracted from virus-free aphids (A−), RhPV-infected aphids (A+), and aphids fed on baculovirus-derived virus particles (A1 and A2). (B) RT-PCR showing aphid-to-aphid transmission of RhPV6 at 17 dpi. Lane M, 1-kb ladder. Templates: total RNA from lanes 1 to 5, progeny aphids from five separate plants harboring aphids fed on baculovirus-derived virus particles; A−, virus-free aphids; A+, known RhPV-infected aphids; +, positive-sense T7 in vitro transcript or RhPV RNA.

baculovirus-derived RhPV6 and not from contamination by uncloned RhPV (data not shown; see also below).

Aphid-to-aphid transmission of RhPV6. To determine the stability of the nonviral sequences and general fitness of the baculovirus-derived RhPV6 genome, RhPV6 was allowed to replicate and accumulate in an aphid colony. The 27-nm-diameter isometric virus particles purified at 47 dpi from aphids fed with baculovirus-derived virus particles were morphologically indistinguishable from RhPV particles (Fig. 5). Moreover, Western blot assays with anti-RhPV antiserum showed the presence of 28-, 29-, and 30-kDa viral capsid proteins in the aphids fed with baculovirus-derived virus particles but not in the virus-free aphids (Fig. 6A). Aphid-to-aphid transmission of RhPV6 was also demonstrated in all five replicate colonies, as determined by RT-PCR at 17 dpi (Fig. 6B).

At 32 days after feeding on virus particles, 5′- and 3′-terminal sequences of the virus genome were determined by RACE. Uncloned and cloned PCR products from 5′ RACE of RhPV RNA from infected aphids were sequenced and revealed a 5′-terminal, vector-derived sequence extending 119 nt upstream of the natural 5′ end of the RhPV RNA (Fig. 1). This is consistent with transcription initiation from the authentic *polh* promoter sequence of the pFastbac1 vector and not from the second core late promoter sequence inserted immediately upstream of the RhPV sequence (Fig. 1). Sequencing of cloned PCR products from 3′ RACE revealed transcripts terminating within the SV40 polyadenylation signal from the pFastBac1 vector in all 20 of the clones sequenced. This indicates that the majority (if not all) of the transcripts had an additional 210 non-RhPV nt downstream of the natural 3′ end of RhPV RNA, excluding the poly(A) tail. We conclude that the cloned RhPV RNA retained substantial portions of vector sequence at each end but the virus remained fit and able to infect, replicate in, and be transmitted between aphids.

DISCUSSION

Baculovirus-mediated RhPV expression in Sf21 cells. We have demonstrated that stable, infectious RhPV can be expressed from a recombinant baculovirus. Baculovirus-produced virus particles resembled wild-type virions, but additional larger virus particles were also seen. AcRhPV6-produced virus particles were localized within the nuclei of baculovirus-infected cells rather than in the cytoplasm, where RhPV virions normally accumulate (20). The nuclear localization of baculovirus-expressed proteins may result from the increased permeability of the nuclear envelope at late stages of baculovirus infection (24). Expression and processing of the RhPV polyproteins required for replication and assembly in Sf21 cells were not unexpected. The RhPV genome contains two IRESs that facilitate cap-independent translation of the replication genes from ORF1 and structural proteins from ORF2 (Fig. 1). The 5′ IRES was previously known to function efficiently in Sf21 cells and in an in vitro Sf21 cell lysate system (29, 40). The intergenic region IRES directs translation initiation from a non-AUG codon and does not require any canonical initiation factors for assembly of initiation complexes on the mRNA (33, 47). Thus, it is not surprising that the genome was translatable in Sf21 cells.

The larger-than-wild-type genomic RNA that accumulated in Sf21 cells is consistent with the inclusion of vector-derived bases at the 5′ and 3′ ends. The first base of the RhPV genome was positioned 119 bases downstream of the *polh* transcriptional start site (Fig. 1). A second *polh* core promoter (ATAAG) was placed immediately upstream of the RhPV coding sequence. It is not surprising that transcription initiated

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from the first *polh* promoter and not the second potential promoter because the latter lacks the upstream and downstream sequences necessary for optimal *polh* promoter activity (32). The 3′ end of the RhPV genome transcript had 210 vector-derived bases, terminating via the SV40 polyadenylation signal as predicted. In summary, the RhPV6 RNA expressed in Sf21 cells and replicated in aphids was 10,340 nt long, comprising 119 upstream nonviral bases, 10,011 bases of the RhPV genome, and 210 downstream nonviral bases.

Replication of RhPV in lepidopteran cells. The presence of negative-sense RNA, and hence probable replication of RhPV RNA, in AcRhPV6-infected cells (Fig. 4B) was intriguing because Sf21 cells do not support the replication of RhPV following transfection with viral RNA (5). However, it is possible that the negative-sense RNA did not serve as a template for subsequent positive-sense RNA synthesis in baculovirus-infected Sf21 cells and that the encapsidated RNA in virus particles was simply the positive-sense RhPV RNA transcribed directly from the baculovirus vector. Negative-strand RNA was also observed on baculovirus expression of *Flock house virus* (28). Full replication of RhPV RNA may occur in Sf21 cells because baculovirus infection may debilitate cellular defense components that would otherwise block RNA virus replication. Alternatively, the large amounts of RNA, and resulting viral proteins, generated from the *polh* promoter may overwhelm host defenses that normally block RhPV replication at a very early stage in RhPV infection. As is the case for other viruses (28), the efficiency of packaging of RhPV6 genomic RNA is expected to be higher if RhPV6 actually replicates in baculovirus-infected cells.

RhPV RNA tolerates hundreds of nonviral bases at its termini. There were no baculovirus mRNAs in test aphids at 32 dpi, as determined by RT-PCR (data not shown), which eliminated the possibility of AcRhPV6 replication in aphids. It is highly unusual for a positive-sense RNA virus to replicate and stably accumulate in a cell with more than 100 nonviral bases at each terminus. Most RNA viruses tolerate few nonviral bases, especially at the 5′ end (2, 8, 46). Moreover, when present on the initial inoculum RNA, such bases are often lost rapidly upon replication (1, 6, 17, 28, 48). Baculovirus-expressed *Flock house virus* RNA2 is another exception in that it tolerated 700 nonviral bases at its 5′ end, although it was not shown to replicate in its natural host (1, 6, 17, 28). Also, recombinant poliovirus has been shown to stably maintain a nonviral insertion of 282 nt (for at least 20 passages) and to maintain a 420-nt insertion for nine passages before being lost by recombination (18). In those cases, however, the extra bases were not at the termini of the genome. Allowance for variation at the extreme 5′ terminus was shown in coxsackie B virus (another picornavirus), in which up to 49 nt from the 5′ end, including part of the cloverleaf needed for replication, was deleted in natural strains that persist in cardiomyocytes (26). Similarly, deletion of the 3′ untranslated region [while retaining the poly(A) tail] reduced, but did not completely inhibit, poliovirus RNA replication (7). Thus, although some RNA viruses tolerate extraneous bases or variations near the termini of the genomic RNA, RhPV is unique in maintaining more than 100 nt of nonviral sequence at each end throughout passages from aphid to aphid for at least 32 days on plants. The sequence requirements for the termini of an RNA virus may be less stringent than previously thought.

The maximum allowable increase in genome length is subject to packaging constraints. In this case, RhPV can still encapsidate a genome that is extended by 329 nt. The extended RhPV RNA was detected within baculovirus-produced virus particles by RT-PCR (Fig. 3), and the infectivity of baculovirus-derived virus particles confirmed that the full-length genomic sequence was present in at least a portion of the virus particles. The efficiency of translation of structural proteins directed by the intergenic region IRES of *Cricket paralysis virus* (*Dicistroviridae*) increases when the cell is under stress, an expected result of virus infection (19, 47). The structural proteins of RhPV are likely to be produced immediately following transcription of the RhPV template by the baculovirus, because the cell has been stressed by baculovirus infection.

A potential aphid control agent. There are several examples of the use of SRVs for insect pest control (11, 42), including the use of *Cricket paralysis virus* for control of the olive fruit fly (30) and *Helicoverpa armigera stunt virus* (*Tetraviridae*) for control of *H. armigera* (12). Baculovirus expression of infectious clones of SRV provides an alternative approach to the production of viruses such as *H. armigera stunt virus*, for which susceptible cell lines have not been identified. In vitro production is preferable to in vivo production for SRVs and avoids problems with product purity that result from production in insects that harbor multiple viruses (13). Baculovirus expression of SRV would also allow the use of well-developed, large-scale baculovirus production systems.

In summary, we have demonstrated that recombinant baculoviruses can be used to generate recombinant RhPV that is infectious to aphids. The baculovirus expression system represents a new tool to investigate dicistrovirus biology and perhaps for large-scale production of dicistrovirus insect pest control agents.

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