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Successful transcription but not translation or assembly of *Solenopsis invicta* virus 3 in a baculovirus-driven expression system

Clare Allen and Steven M. Valles*

Abstract

Solenopsis invicta virus 3 (SINV-3) is an unclassified positive stranded RNA virus whose characteristics are amenable to development as a microbial insecticide or as a classical biological control agent for the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Bait formulations containing SINV-3 have been produced and were used successfully to transmit the infection and cause significant mortality to fire ant colonies in the laboratory. Unfortunately, there is no available means of propagating infectious SINV-3 particles in vitro, and this has hampered its evaluation and development as a microbial insecticide. In this study, we attempted to utilize a baculovirus expression system as reported by others to produce SINV-3 infectious particles in quantity. A full-length copy of the SINV-3 genome was assembled successfully from 3 overlapping fragments, and a heterologous bacmid-SINV-3 vector (BAC_SINV-3) was produced. The insect cell line Sf21 was transfected with purified high-molecular weight BAC_SINV-3 and supported production of baculovirus expressing full-length SINV-3 transcripts. Despite successful transcription of the SINV-3 genome, evidence that translation was occurring could not be produced. Western analyses for the SINV-3 VP-2 capsid protein were consistently negative.

Key Words: *Solenopsis invicta*; unassigned virus; expression; biological control

Resumen

El virus *Solenopsis invicta* 3 (SINV-3) es un virus ARN de cadena positiva no clasificada, cuyas características son promisorias para el desarrollo de un insecticida microbiano o como agente de control biológico clásico de la hormiga de fuego roja importada, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Se han producido y se han utilizado con éxito formulaciones de cebo que contienen SINV-3 para transmitir la infección y causar una mortalidad significativa en las colonias de hormigas de fuego en el laboratorio. Desafortunadamente, no hay medios disponibles para propagar partículas de SINV-3 infecciosas in vitro, lo que ha hecho más difícil su evaluación y su desarrollo como un insecticida microbiano. En este estudio, hemos intentado utilizar un sistema de expresión de baculovirus según lo informado por otros para producir partículas infecciosas de SINV-3 en cantidad. Se produjo con éxito una copia de longitud completa del genoma SINV-3 de 3 fragmentos sobrepuestos, y se produjo un vector heterólogo bácmido-SINV-3 (BAC_SINV-3). La línea celular de insecto, Sf21, se transfirió con BAC_SINV-3 purificada de alto peso molecular que apoyó la producción de baculovirus que expresan líneas enteras de transcripciones de SINV-3. A pesar de la transcripción con éxito del genoma SINV-3, evidencia de que la traducción estaba ocurriendo no se puede producir. El análisis occidental para la proteína cápside VP-2 de SINV-3 fue consistentemente negativo.

Palabras Clave: *Solenopsis invicta*; virus no asignados; expresión; control biológico

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), was introduced into the United States in the 1930s near Mobile, Alabama (Callcott & Collins 1996), from the Mesopotamian flood plain near Formosa in northern Argentina (Caldera et al. 2008). Currently, *S. invicta* principally occupies the southeastern region of the USA with colonies found as far north as Virginia and west to California (Callcott & Collins 1996; Williams et al. 2001; Allen et al. 2010). Within the last decade, the range occupied by *S. invicta* has expanded globally into Asia (Wong & Yuen 2005), Australia (McCubbin & Weiner 2002), and the Caribbean (Wetterer 2013).

Efforts to expand the biologically-based repertoire of control agents available for *S. invicta* has led to the discovery of 3 fire ant viruses, *Solenopsis invicta* virus 1 (SINV-1), SINV-2, and SINV-3 (Valles et al. 2004, 2007; Valles & Hashimoto 2009). Although all of these viruses possess a positive-sense, single-stranded RNA genome, the genome architecture and effects of each on fire ants are distinctly different.

The SINV-1 and SINV-3 genomes contain 2 large open reading frames (ORFs), whereas SINV-2 contains 4 ORFs. In addition to genomic architectural differences, the impact of each virus on *S. invicta* is unique. SINV-1- and SINV-2-infected ant colonies do not exhibit any observable symptoms; both viruses appear to cause an asymptomatic, chronic infection. Conversely, SINV-3 is consistently associated with a systemic infection resulting in significant larval and adult mortality (Valles 2012; Porter et al. 2013; Valles et al. 2013). Indeed, bait formulations containing SINV-3 have been produced and were successfully used to transmit the infection to uninfected colonies in the laboratory (Valles et al. 2013) and field (Valles & Oi 2014). Thus, SINV-3 appears to be an excellent candidate for development as a microbial insecticide or as a classical biological control agent in areas where the virus is absent (Yang et al. 2010). The SINV-3 genome is organized with 2 large, non-overlapping ORFs (Valles & Hashimoto 2009); the 5'-proximal ORF encodes the non-structural proteins and the 3'-proximal ORF encodes

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the structural proteins. Recently, Valles et al. (2014a) showed that transcription of SINV-3 proceeds through a frame-shifting mechanism.

At present, there is no available means of propagating infectious SINV-3 particles in vitro, which has hampered its evaluation and development as a microbial insecticide. However, Pal et al. (2007) successfully produced a similar virus, *Rhopalosiphum padi virus* (RhPV) (Picornavirales: Dicistroviridae), in quantity with a recombinant baculovirus system capable of expressing the entire genome of the virus in surrogate insect cells. As there is no available cell line for fire ants, and the method had been established for a similar virus, baculovirus-driven expression seemed to be a viable solution to the production of SINV-3.

In this study, our objective was to utilize a baculovirus expression system as reported by Pal et al. (2007) to generate and produce SINV-3. Although unsuccessful at producing assembled virus particles, the SINV-3/bacmid construct did yield full-length transcripts of the SINV-3 genome from infected Sf21 cells. Recently, Pal et al. (2014) published a corrigendum to their original work (Pal et al. 2007) in which they discuss their inability to repeat the expression of RhPV. Indeed, the authors apparently never successfully expressed RhPV with the baculovirus system (Pal et al. 2014). This new information compelled us to make the scientific community aware of our own failure to manufacture SINV-3 in a surrogate system. Furthermore, we share our difficulties experienced in this attempt, and we discuss peculiarities with SINV-3.

Materials and Methods

CELLS AND VIRUSES

A *Spodoptera frugiperda* 21 (Sf21) cell line was obtained from Invitrogen (Carlsbad, California, USA). Cells were propagated in tissue culture medium TC100 + 10% fetal calf serum (FCS) or SF900II and incubated at 27 °C. The polyhedrin-negative virus AcRP23.lacZ served as a negative control for comparative studies (Possee & Howard 1987).

Construction of the SINV-3 Recombinant pFastBac Plasmid

A SINV-3_pFastBac construct was created with the Bac-to-Bac expression system (Invitrogen). The wild-type SINV-3 genome is a 10,411 bp (GenBank accession number: NC_012531) positive-sense, single-stranded RNA molecule. Difficulties with reverse transcription, insertion, and retention of the entire genome of SINV-3 into the pFastBac vector required that construction of the SINV-3 genome take place by concatenation of sections from sub-clones. Total RNA was extracted from a sample of SINV-3-infected *S. invicta* collected in Gainesville, Florida, USA, using Trizol (Invitrogen) reagent. cDNA was synthesized using gene-specific primers just downstream of the desired section to be amplified and cloned (Valles & Hashimoto 2009). Three overlapping genome fragments generated by reverse-transcriptase polymerase chain reaction (RT-PCR) (Fig. 1) were ligated separately into the TOPOXL vector (Invitrogen). Top10 competent cells were transformed with the ligated plasmids and insert-positive clones grown overnight to obtain sufficient quantities of each plasmid for restriction enzyme digestion and assembly.

The 1st cloned fragment (F1) of the SINV-3 genome (Fig. 1) was produced using oligonucleotide primers p903SalI (5'GTCGACATAAGTAGGAAATTAAGTCCAGTAAGGTTACTGGCATTCT [This oligonucleotide primer was appended with a SalI restriction site (underlined) and the introduced late gene polyhedrin core promoter sequence, ATAAG (bold)]), and p738 (5'ACGAGCTGTGAACTACCAAGAATCCAACGTT) with cDNA being generated using oligonucleotide primer p738.

The 2nd fragment (F2) was produced using primers p770 (5'TGATG-GTGATTATGTTTATATTTCTGAGCATAAAATTC) and p790KpnI (5'GGTACC^{KpnI}ATCCCATTTGAGCAAATGGTTTACCTCCAACCTCAA) with cDNA being generated using gene-specific primer p794 (5'GTTTGAAAACTTTACATTATGATTAATCTTCGCACCTC). The last fragment (F3) of the SINV-3 genome (Fig. 1) was produced by PCR with oligonucleotide primers p902KpnI (5'GGTACC^{KpnI}ACTTTACATTATGATTAATCTTCGCACCTCCAC) and p730 (5'CCAAAATTTGAAACTATTGAAACATTATCTTTTATA) with cDNA generated from gene-specific primer p794 (5'GTTTGAAAACTTTACATTATGATTAATCTTCGCACCTC). Concatenation of each of these fragments was accomplished by excision from the XL vector with appropriate restriction enzymes and subsequent ligation into the pFastBac vector and transformation of TOP10 competent cells.

F2 and pFastBac1 (Invitrogen) were double digested with *SpeI* and *KpnI*. The 5,402 nt SINV-3 fragment and the linearized pFastBac1 vector were gel purified and ligated for 18 h at 16 °C. The ligated vector was transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen). The resulting construct (pFastBac1_F2 [genome position 3,515–8,917]) and sub-clone F3 (genome position 6,296–10,377) were digested with *ApaI* and *KpnI*. F3 and the linearized pFastBac1_F2 construct were gel purified, ligated, and transformed into One Shot TOP10 chemically competent *E. coli* cells (Invitrogen). The clone denoted construct pFastBac1_F2 + F3 (Fig. 1) contained the SINV-3 genome from position 3,515 bp (*SpeI* site) to 10,377 bp minus the polyadenylated tail. F1 was originally digested with *BamHI/SalI*, and gel purified before being further digested with *SpeI* to release a fragment that would also be gel purified. The 5' end *SalI-SpeI* gel-purified fragment was ligated into pFastBac1 overnight at 16 °C before being transformed into TOP10 *E. coli* cells (pFastBac1_F1). The pFastBac1_F1 construct containing the 5' end of SINV-3 and pFastBac1_F2+F3 were double digested with *SpeI* and *KpnI* and the fragments gel purified. The gel-purified pFastBac1_F1 and F2 + F3 fragments were ligated overnight at 16 °C before being transformed into TOP10 *E. coli* cells. The final construct, denoted pFastBac1_SINV-3 (Fig. 1) was confirmed by restriction enzyme digestion, PCR of the entire genome, and sequencing analysis of the 5' and 3' termini and the ligation junctions.

Full-length clones were chosen on the basis of complete amplification of the entire SINV-3 genome with primers p902 (5'ACTTTACATTATGATTAATCTTCGCACCTCCAC) and p903 (5'TAGGAAATTAAGTCCAGTAAGGTTACTGGCATTCT) using LA Taq DNA Polymerase (TaKaRa Bio Inc., Mountain View, California, USA) and denoted pFastBac1_SINV-3.

Production of Recombinant Baculoviruses

The construct, pFastBac1_SINV-3, was transformed into DH10Bac *E. coli* cells according to the manufacturer's instructions using the Bac-to-Bac Baculovirus Expression System (Invitrogen) with the exception that prior to spreading onto the agar plate the cultures were centrifuged at 1,200 × g for 5 min and re-suspended in 175 µL SOC medium. Insert-positive clones (by PCR amplification) were re-streaked onto Luria broth agar plates with 50 µg/mL ampicillin and permitted to grow for 48 h at 37 °C. After growing for approx. 40 h at 37 °C with shaking, the high-molecular weight bacmid DNA containing the assembled SINV-3 genome was purified using the Purelink HiPure Plasmid DNA Miniprep kit according to the manufacturer's instructions (Invitrogen).

Recombinant viruses were produced by transfections of purified bacmid DNA (2.0 µg BACSINV-3 per dish) with cellfectin (Invitrogen) into 35 mm dishes of Sf21 cells seeded at 8 × 10⁵ per dish. Dishes were left overnight at 27 °C before removing the transfection medium and replacing with 2 mL of Baculogold medium (BD Biosciences, San Jose, California, USA) + 10% FCS and 5% streptomycin/penicillin. Both the cell pellet and supernatant of the transfection experiment (P1 stock)

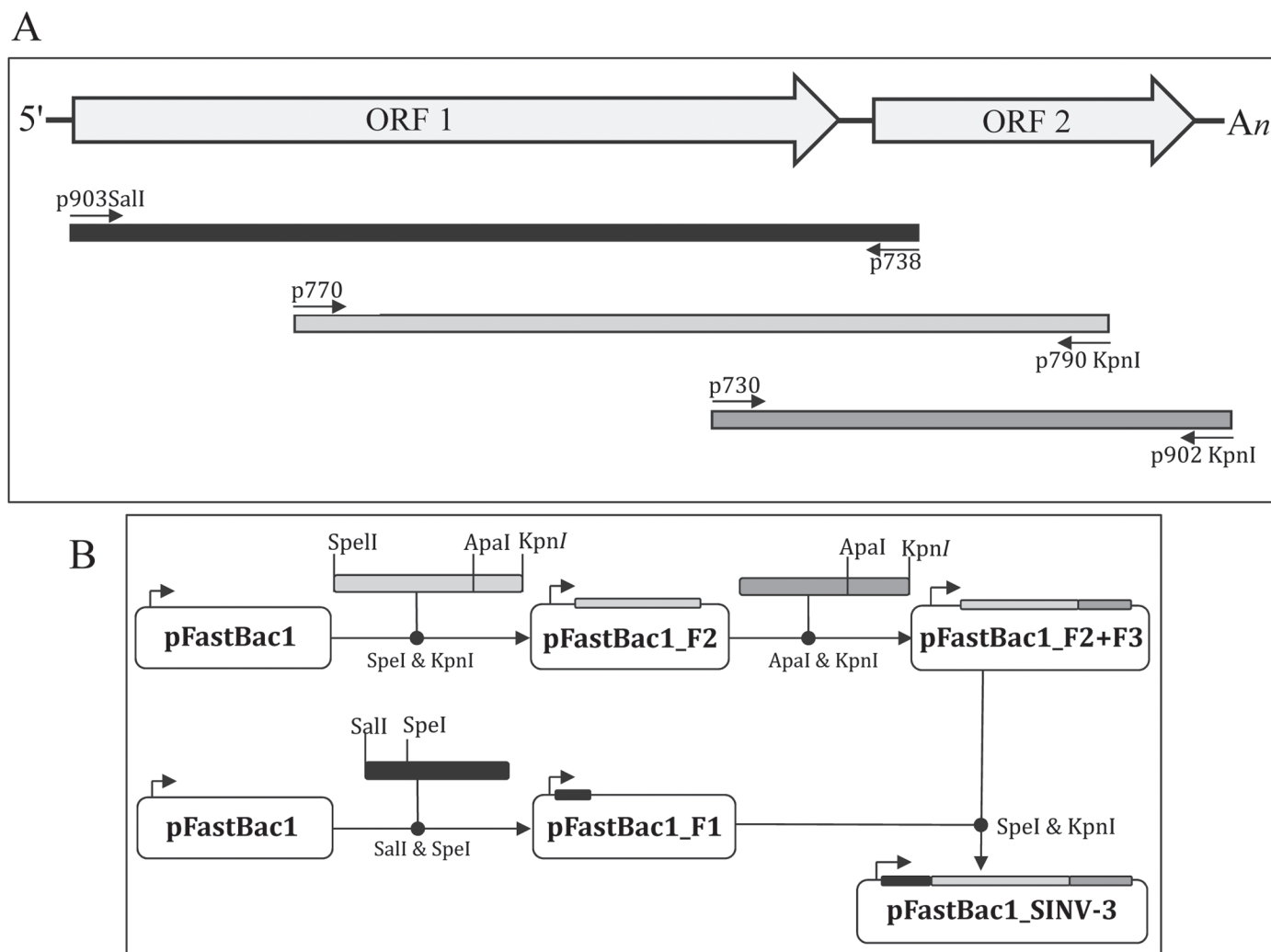


Fig. 1. Schematic of the SINV-3 genome and sub-cloning strategy to assemble the pFastBac1 donor plasmid/SINV-3 construct. (A) Organization of the SINV-3 genome illustrating the 2 ORFs numbered 1 and 2 that encode for non-structural and structural proteins, respectively, and the genome sections sub-cloned. Oligonucleotide primers used to generate cDNA and amplify each section are indicated. Primers with introduced restriction sites are also indicated. (B) Unique restriction sites for each sub-clone and the assembly process employed to concatenate the entire SINV-3 genome in the pFastBac1 donor vector.

were harvested at 5 d post infection (dpi) by centrifugation at 3,300 rcf for 5 min, and then the pellet was washed twice with phosphate buffered saline (PBS).

Viable infectious recombinant AcSINV-3 virus particles were detected through plaque assay in *Sf*21 cells using standard procedures and stained using neutral red. Individual plaques were picked using sterile glass Pasteur pipettes and placed into 400 μ L SF900II + 10% FCS. Further amplified stocks were prepared in *Sf*21 cells from either the P1 stock or the individual plaque picks.

Successful transfections were verified by PCR of the entire SINV-3 genome with oligonucleotide primers p902 and p903 using LA Taq DNA Polymerase as described above.

Detection of AcSINV-3 Transcripts

To detect transcripts generated in AcSINV-3-infected *Sf*21 cells, total RNA was extracted by the Trizol method. Nucleic acid purity and concentration were determined by absorbance (Nanodrop spectrophotometer). Because of difficulties with reverse transcribing the entire SINV-3 genome (wild type or recombinant), 3 regions of the SINV-3 genome were reverse transcribed and amplified to verify transcript production.

The 1st region was at the 3' terminus, which included some of the SV40 poly(A) signal sequence from the pFastBac1 vector. The reverse transcription oligonucleotide primer used was A07 (5'GGTGTGGGAGGTTTTTAAGCAA). PCR was subsequently conducted using oligonucleotide primers A07 and p813 (5'AAGAGAACGTATGCCTACTCCATCAGAAGCAT) with an annealing temperature of 57 $^{\circ}$ C. The 2nd region was near the 5'-proximal end of ORF1. Reverse transcription was completed with oligonucleotide primer p752 (5'AATCCATTTACATCGGGTAAAGTAAGAAGTCTCTGCT). PCR was subsequently conducted using oligonucleotide primers p752 and p769 (5'GCAAATGCTACTAATCTTAAATGGATCAGAGG) with an annealing temperature of 61 $^{\circ}$ C. The final region was at the 3'-proximal end of ORF2 and completed by quantitative PCR (qPCR) as described previously (Valles & Hashimoto 2009). In each case, total RNA was digested with DNase (Sigma Chemical Co., St. Louis, Missouri, USA).

Detection of AcSINV-3 Capsid Protein

*Sf*21 cells transfected with the AcSINV-3 were probed by Western blot analysis for production of SINV-3 capsid proteins. Cells were collected by centrifugation, and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20% gradient) and

subsequently electroblotted onto a polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, California, USA) for 2 h at 350 mA. Polyclonal antibodies were developed to a region of the SINV-3 capsid protein (VP2; epitope ERMPTPSEAYDNSK) in a rabbit host by GenScript USA, Inc. (Piscataway, New Jersey, USA) according to the company's standard protocols. Western blot analysis was conducted with the SINV-3-polyclonal antibody (pAb) preparation at a 10,000-fold dilution. Briefly, the electroblotted PVDF membrane was blocked in Tris buffered saline (TBS ; 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) + 1% bovine serum albumin (BSA) for 1 h. Primary antibody (SINV-3-pAb) was added to the TBS + 1% BSA solution for 2 h at room temperature with shaking (40 rpm). The membrane was rinsed twice with TTBS (TBS + 0.05% Tween 20), probed with secondary antibody (10,000-fold dilution), goat anti-rabbit conjugated with alkaline phosphatase (Sigma, St. Louis, Missouri, USA) for 1 h, and rinsed twice with TTBS. The membrane was incubated for several minutes with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium for the colorimetric detection of alkaline phosphatase activity. Once bands were detected on the blot, the reaction was terminated by rinsing the membrane with deionized water 3 times. Positive controls from wild-type SINV-3 infections were included in each evaluation.

Results and Discussion

Difficulties in reverse transcribing the entire genome and ligating the full-length SINV-3 cDNA into a vector forced us to use an alternative method to produce a full-length clone of the SINV-3 genome. A full-length copy of the genome was assembled successfully from 3 overlapping fragments (Figs. 1 and 2). Assembly was achieved in the pFastBac1 donor plasmid by exploiting unique restriction sites to concatenate the entire genome from

3 sub-clones. Assembly was verified by PCR amplification of the full-length genome from the plasmid DNA template. The sequence of the pFastBac1_SINV-3 construct was verified by PCR amplification of the 5' and 3' termini, the restriction enzyme/ligation sites, and the presence of the polyhedrin promoter region upstream of the 5' end of the SINV-3 genome (Fig. 2). Thus, the SINV-3 genome was properly assembled into the pFastBac1 donor plasmid; in silico analyses of these amplified regions yielded start and stop codons, and ORFs identical to the wild-type virus.

The completed transfer vector, denoted pFastBac1_SINV-3 was composed of the SINV-3 genome (10,377 bp in length) and pFastBac1 vector. Upstream of the SINV-3 sequence lay the late gene polyhedrin promoter with a wild-type start codon (ATG) mutated to ATT so that translation would occur at the ATG site of the SINV-3 genome (Fig. 2). The inserted SINV-3 construct differed slightly from the original sequence reported (Valles & Hashimoto 2009). First, the 8-most nucleotides at the 5' terminus of the original sequence were not included because of difficulties amplifying this region from wild-type virus. Second, at the 3' terminus, 9 nucleotides preceding the polyadenylated region were excluded. This region was part of the 3' untranslated region (UTR) and was replaced with a *KpnI* restriction site followed by an SV40 polyadenylation signal. Positive-strand RNA viruses exhibit comparatively higher mutation rates because of the lack of proofreading machinery, template switching, and other factors which could have contributed to our difficulties. However, previous use of this method with *Rhopalosiphum padi* virus (RhPV) showed that non-virus nucleotide insertions at the 5' and 3' UTR termini had no effect on the ability to produce infectious virus (Pal et al. 2007). Whether our deletions had an impact on translation was not determined. Indeed, in light of the corrigendum recently published by Pal et al. (2014), these small changes at the virus termini may have had more of an impact than thought previously.

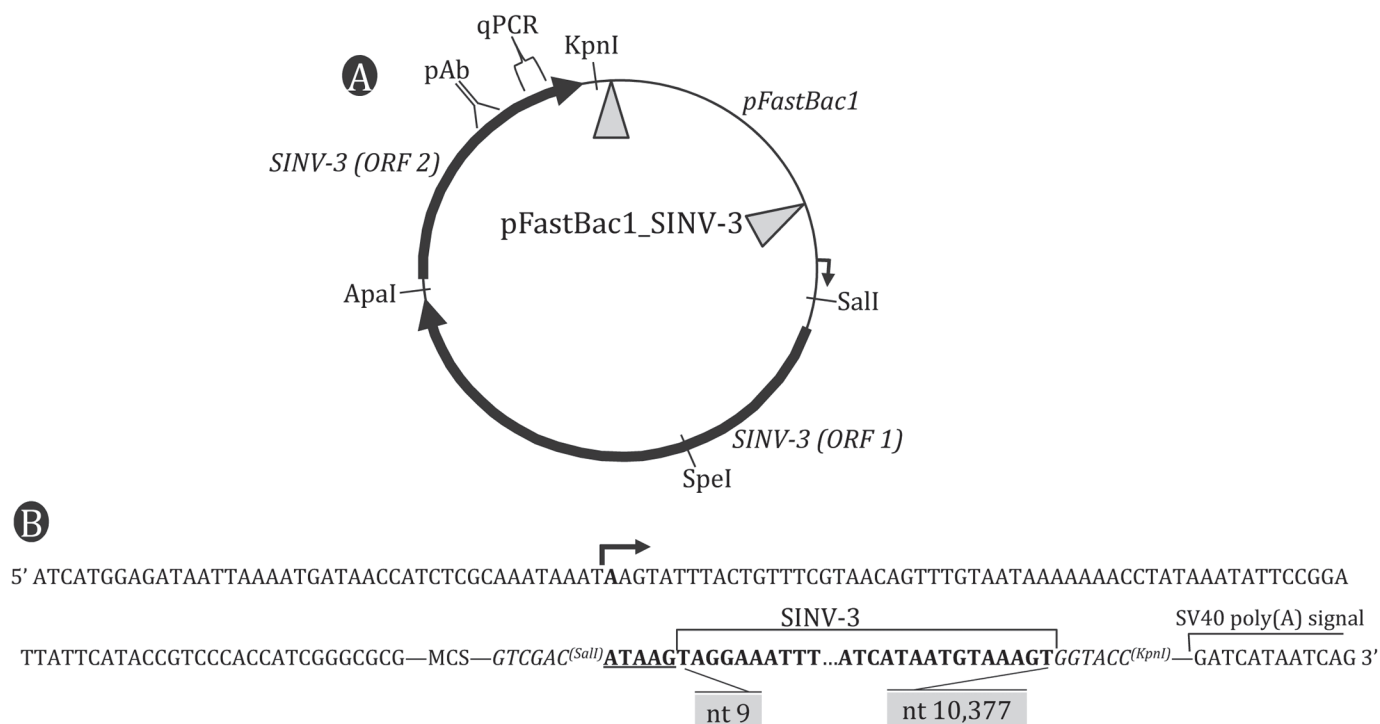


Fig. 2. (A) pFastBac1_SINV-3 hybrid construct map. Locations of the restriction sites (black hash marks), bacterial transposon Tn7 sites (grey triangles), polyhedrin promoter (angled arrow corresponding to the sequence below), SINV-3 open reading frames (dark closed arrows), and approximate location of the area detected by the polyclonal antibody preparation (pAb) are shown. (B) Verified sequence of the pFastBac1_SINV-3 hybrid construct illustrating the late gene polyhedrin promoter (angled arrow). SINV-3 sequence is in bold font, with pFastBac1 sequence in normal font, and restriction sites are superscripted and corresponding sequences italicized. Underlined sequence represents the inserted late gene polyhedrin core promoter. Analyzed sequences of the 5' and 3' termini and restriction sites were identical to the wild-type virus.

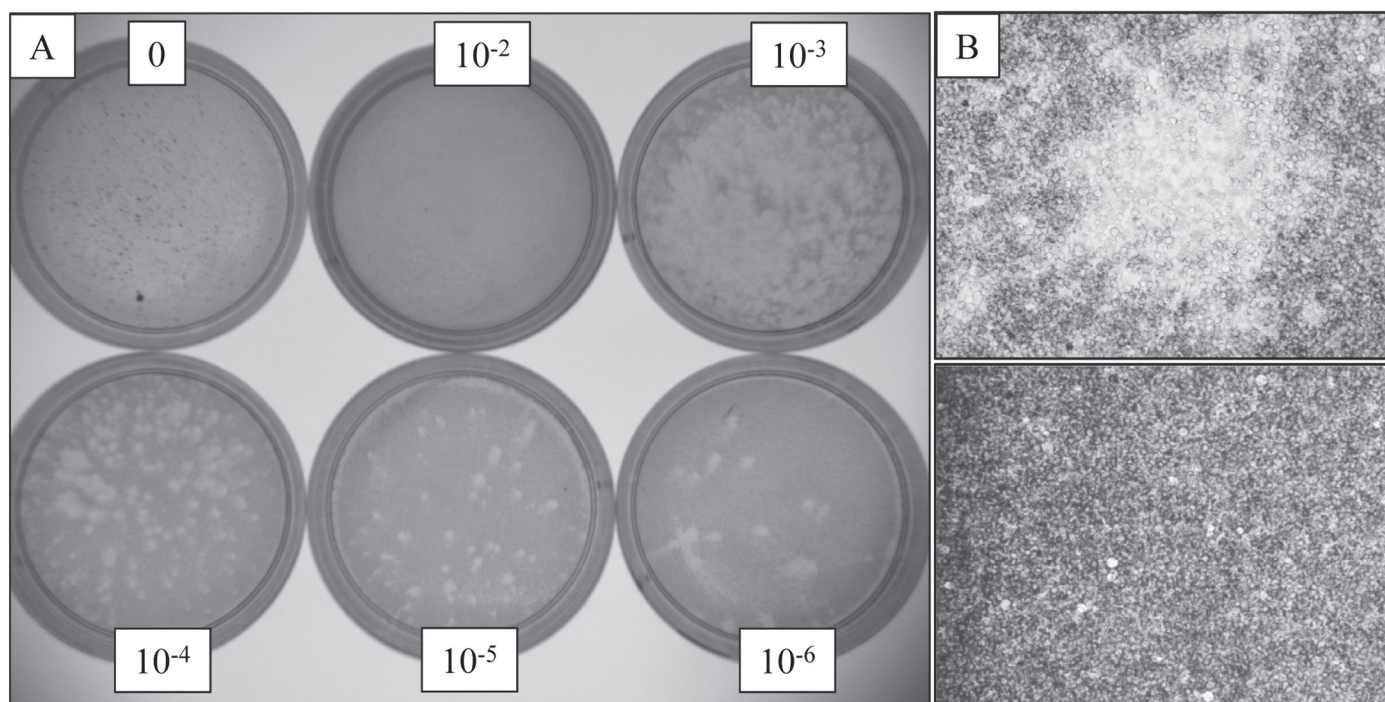


Fig. 3. (A) Plaque assay results for recombinant SINV-3 (AcSINV-3) transfection of *Sf21* cells indicating the dilution used for each plate. (B upper panel) Representative plaque with *Sf21* cells stained with neutral red 10 d after transfection (magnified 100 times). (B lower panel) Corresponding mock-infected *Sf21* cells (negative control) after 10 d of exposure. Plaque areas identify infection of insect cells by virus with corresponding cell death.

After 10 d of exposure, very small and irregular-shaped plaques were observed in the AcSINV-3-infected dishes (Fig. 3A). Light microscopy and neutral red staining confirmed the presence of plaque formation with a central clearing surrounded by live (red) cells (Fig. 3B, upper panel). The polyhedrin-negative, AcRP23.*lacZ* virus also produced plaques of similar size. In comparison, uninfected *Sf21* cells contained a layer of live red cells free of plaque formation (Fig. 3B, lower panel).

Plaques were taken at random and amplified by infecting additional dishes of *Sf21* cells. These stocks were subsequently evaluated to determine whether transcription and translation of SINV-3 was occurring. RNA treated with DNase I was prepared from AcSINV-3-infected *Sf21* cells revealing an increase in SINV-3 transcript production over 5 d (Fig. 4). Maximum transcript production appeared to occur at 4 dpi. These results clearly demonstrated that baculovirus-driven expression of the SINV-3 genome was occurring. Indeed, this result was confirmed by amplification of the 3' (Fig. 5A) and 5' termini (Fig. 5B) from RNA templates purified from AcSINV-3-infected *Sf21* cells. It appeared that the SINV-3 transcript was retained within the *Sf21* cells based on more intense PCR amplification in RNA preparations obtained from pelleted *Sf21* cells compared with the soluble fraction (Fig. 5A). Successful transposition of the entire SINV-3 genome into the bacmid DNA was verified by amplification of the entire genome from *Sf21*-infected cells (Fig. 5C). Despite successful transcription of the SINV-3 genome, evidence that translation was occurring could not be obtained. Western blot analysis for the VP-2 protein (SINV-3 capsid protein) was consistently negative (Fig. 5D) as was electron microscopy using negative staining and Western blot analysis with an antibody preparation to the RdRp (data not shown). The level of detection for VP-2 had been determined empirically to be 100 genome equivalents (Valles et al. 2014b). Based on qPCR data of the time-course expression, the Western blotting limit of detection was sufficient enough to detect VP-2 had translation occurred.

SINV-3 has been shown to be an effective biologically-based control agent for the invasive red imported fire ant, *S. invicta* (Porter et al. 2013; Valles et al. 2013). However, commercial utilization of the virus

requires a method to mass produce it. Thus, we were quite excited to read about the successful production of RhPV in a baculovirus-driven system (Pal et al. 2007). Although we were able to successfully drive the expression of SINV-3, translation and assembly of SINV-3 were never observed. Initially, Pal et al. (2007) reported RhPV transcription and virus assembly in their baculovirus-driven system. The heterologously-expressed RhPV was even able to infect the aphid host and was stable over time. Unfortunately, these results could not be repeated, and

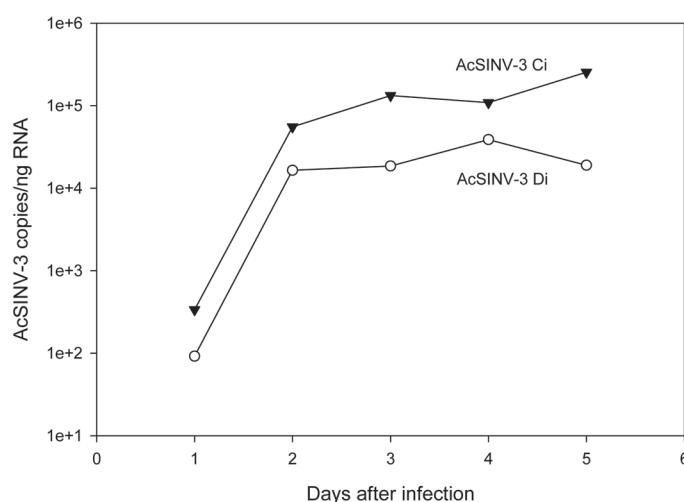


Fig. 4. Quantitative PCR (absolute) results evaluating transcript production of SINV-3 by AcSINV-3-infected *Sf21* cells. RNA preparations from AcSINV-3 (2 replicates, Ci and Di) were treated with DNase I, reverse transcribed, and amplified by qPCR. Results were compared with a series of plasmid constructs containing a portion of the SINV-3 genome (10^2 – 10^9 genome equivalents). The region amplified was at the 3'-most end of ORF2 (see Fig. 2). No amplification was detected in polyhedrin-negative AcRP23.*lacZ* preparations.

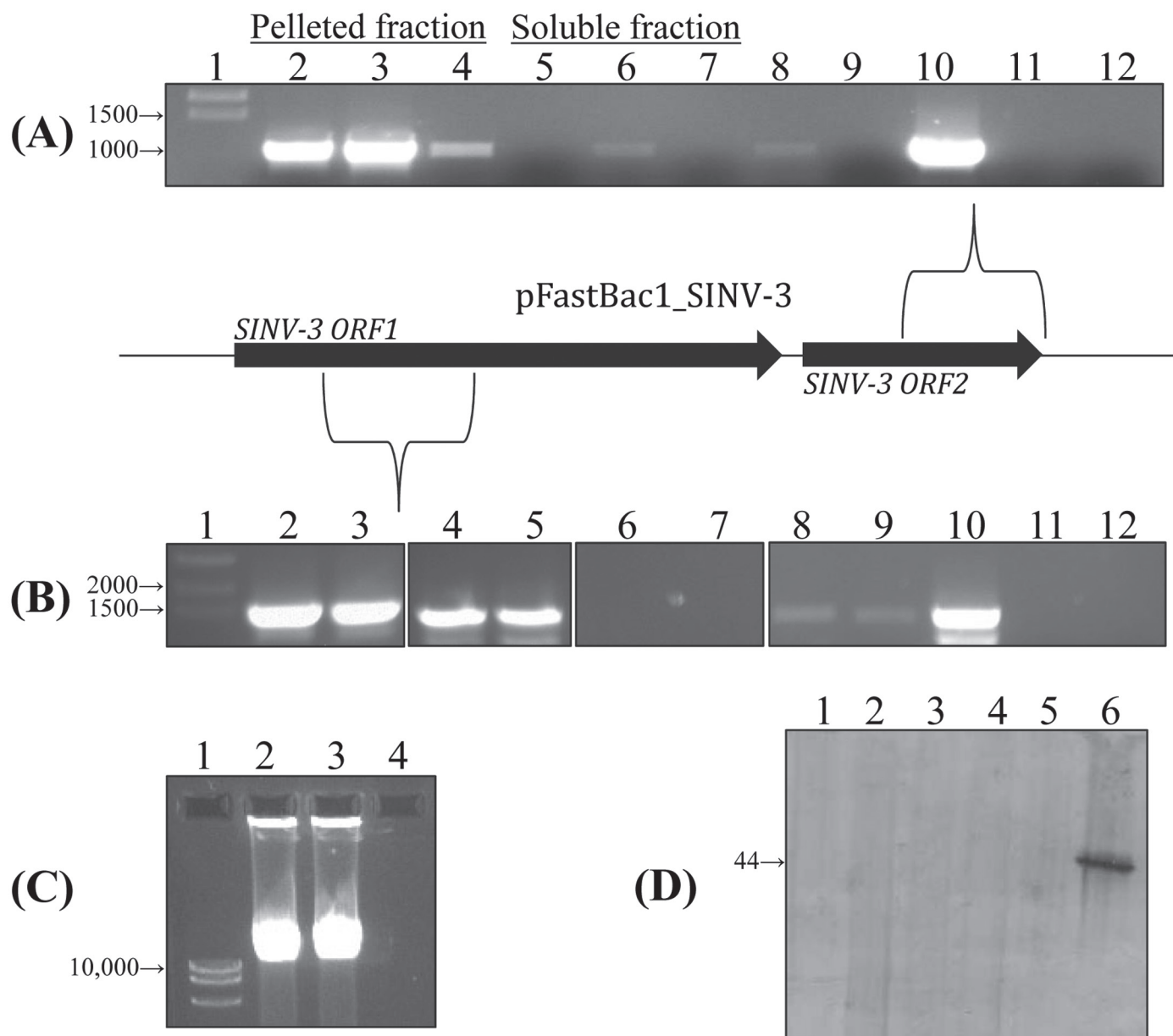


Fig. 5. Confirmation of heterologous expression of SINV-3 transcript by amplification of the 3' (A) and 5' termini (B) from RNA templates purified from SINV-3-transfected Sf21 cells. Regions amplified are illustrated in the genome diagram between (A) and (B). **(A)** Three plaque preparations (AcSINV-3 CiC, AcSINV-3 CiD, and AcSINV-3 DiD) were separated by centrifugation into soluble and pelleted fractions, treated with DNase I, reverse transcribed, and the 3' end of the genome amplified by PCR. Lane assignments were as follows: 1 = mass marker (bp); 2, 6 = AcSINV-3 CiC; 3, 7 = AcSINV-3 CiD; 4, 8 = AcSINV-3 DiD; 5, 9 = mock infection; 10 = positive control (wild-type virus); 11 = negative control; 12 = non-template control. **(B)** AcSINV-3 plaque preparations (CiC and DiD) evaluated by PCR of the 5' end of the genome (RNA preparations). Lane assignments were as follows: 1 = mass marker (bp); 2, 3 = DNase treated, reverse transcribed; 4, 5 = without DNase treatment, reverse transcribed; 6, 7 = DNase treated, without reverse transcription; 8, 9 = without DNase treatment, without reverse transcription; 10 = positive control; 11 = negative control; 12 = non-template control. **(C)** PCR amplification of the entire SINV-3 genome from DNA preparations of AcSINV-3 CiC (lane 2) and AcSINV-3 DiD (lane 3). Lane 1, molecular markers; lane 4, non-template control. **(D)** Western blot to evaluate translation of the SINV-3 transcript by detection of viral capsid protein 2 (VP2). Lane assignments were as follows: 1 = AcSINV-3 CiC (4 dpi); 2 = AcSINV-3 CiC (5 dpi); 3 = AcSINV-3 CiD (4 dpi); 4 = AcSINV-3 DiD (5 dpi); 5 = mock infection (negative control); 6 = positive control (purified wild-type SINV-3; kDa).

these authors recently published a corrigendum in which they report the baculovirus-expressed RhPV was not infectious (Pal et al. 2014).

Although we did not achieve our objective of heterologous production of SINV-3, we felt compelled to publish these results, especially in light of the corrigendum published by Pal et al. (2014). Additional characterization of SINV-3 has revealed that it produces its capsid proteins from a subgenomic RNA via a frameshifting mechanism (Valles et al. 2014a). Indeed, the virus exhibits characteristics more consistent with Caliciviruses than Dicistroviruses. A more complete understand-

ing of the expression mechanisms of SINV-3 may have been a prudent requirement before attempting expression of the virus.

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