A cell culture system and production of an infectious clone of Rhopalosiphum padi virus (Dicistroviridae)

Sandhya Boyapalle

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Cell Biology Commons, and the Microbiology Commons

Recommended Citation

Boyapalle, Sandhya, "A cell culture system and production of an infectious clone of Rhopalosiphum padi virus (Dicistroviridae)" (2005). Retrospective Theses and Dissertations. 1324.

https://lib.dr.iastate.edu/rtd/1324

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
A cell culture system and production of an infectious clone of

*Rhopalosiphum padi* virus (Dicistroviridae)

by

Sandhya Boyapalle

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Microbiology

Program of Study Committee:

Bryony C. Bonning, Co-major Professor
W. Allen Miller, Co-major Professor
Gwyn A. Beattie
Larry J. Halverson
Gregory J. Phillips

Iowa State University
Ames, Iowa
2005
This is to certify that the doctoral dissertation of

Sandhya Boyapalle

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

For the Major Program
TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION 1

CHAPTER 2. IDENTIFICATION OF INSECT CELL LINES SUSCEPTIBLE TO 
*RHOPALOSIPHUM PADI* VIRUS (DICISTROVIRIDAE) 19

CHAPTER 3. GENERATION OF *RHOPALOSIPHUM PADI* VIRUS-LIKE 
PARTICLES USING THE BACULOVIRUS EXPRESSION SYSTEM 47

CHAPTER 4. INFECTIOUS *IN VITRO* TRANSCRIPTS OF *RHOPALOSIPHUM PADI* VIRUS SYNTHESIZED FROM A FULL-LENGTH cDNA 
CLONE 70

CHAPTER 5. GENERAL CONCLUSIONS 97

REFERENCES 104

ACKNOWLEDGEMENTS 115
ABSTRACT

*Rhopalosiphum padi* virus (RhPV) was first isolated from the bird cherry-oat aphid, *Rhopalosiphum padi*. RhPV, an icosahedral virus, belongs to the family *Dicistroviridae*. It has a 10 kb positive-sense RNA genome, with two viral open reading frames (ORFs). We have identified two cell lines, Z10-2 and DMII derived from the homopterans, *Homalodisca coagulata* and *Dalbulus maidis* that are permissive for RhPV. Infection, viral replication and production of virions was confirmed by northern blot hybridization, RT-PCR, western blot analysis and immunoelectron microscopy. A cell culture system that allows replication of the virus is crucial for further study of the biology of RhPV.

The long term goal of this project is to produce aphid resistant transgenic plants that express RhPV. Acquisition of an infectious clone of RhPV is of primary importance for development of such an aphid resistance technology. A full-length cDNA clone of RhPV was constructed using overlapping reverse transcription PCR products. RNA transcribed from the full-length cDNA clone was infectious upon transfection into Z10-2 and DMII cells, resulting in production of progeny virus phenotypically indistinguishable from the parent virus. The *in vitro* transcript caused the same cytopathic effects as those caused by transfection of cells with the viral RNA. The virus-like particles (VLPs) purified from the cells transfected with the full-length transcript were infectious to Z10-2 cells and also to *R. padi*. The infectious cDNA clone of RhPV, together with the cell culture system, will provide valuable experimental tools for the study of replication and pathogenesis of RhPV.

The recombinant baculovirus AcRhPV6, which contains the cDNA of RhPV under the control of the polyhedrin promoter, was constructed using the Bac-to-Bac baculovirus
expression system. Expression of the AcRhPV6 in Sf21 cells resulted in formation of RhPV VLPs, whose capsids are structurally and immunologically indistinguishable from the native virions. These results show that recombinant baculoviruses can be used to generate immunogenic VLPs of RhPV with some degree of infectivity to aphids. The baculovirus expression system represents an alternative tool for use in the study of the assembly and structure of viruses.
CHAPTER 1.

GENERAL INTRODUCTION

Aphids are among the most pervasive pests of temperate agriculture and affect almost all agricultural crops. Invasive species such as the Russian wheat aphid and the more recently introduced soybean aphid have had a particularly severe impact on U.S. agriculture. Economic losses result from direct feeding on plants, from aphid-transmitted plant viruses, and from production of honeydew which results in growth of harmful sooty molds.

Management of aphid populations has relied largely on the use of environmentally damaging, classical chemical insecticides. However, aphids readily develop insecticide resistance. An alternative approach for aphid management would be use of insect-specific toxic polypeptides. Although a number of highly effective peptide toxins that act within the insect hemocoel have been identified, it has not been possible to exploit these toxins for aphid control because of the lack of an appropriate delivery system. Because the aphid virus, *Rhopalosiphum padi* virus (RhPV) infects and replicates in aphids, it may provide an appropriate delivery system for insect toxins that are active within the aphid hemocoel. The development of aphid resistant transgenic plants would contribute to environmentally sustainable pest management and would confer significant economic benefit to U.S. agriculture.

RhPV infects several species of aphid, including the Russian wheat aphid and vectors of Barley yellow dwarf virus (BYDV), but host range studies of RhPV have been limited. RhPV is a member of the emerging *Dicistroviridae* family. These viruses are widespread among insects and other invertebrates, as new dicistroviruses are frequently being
discovered. To understand the biology of these viruses and to exploit them for insect control purposes, an infectious clone of the genome is essential.

Aphid control

Current aphid control continues to rely heavily on the use of chemical insecticides (Schepers, 1989). In addition to the potentially deleterious impact of these pesticides on non-target organisms (Flickinger et al., 1991), the efficacy against aphids can be short-lived with the rapid evolution of insecticide resistance (Devonshire, 1989). Because of the pervasive nature of aphid damage on a wide variety of crops, the study of aphid resistance genes has received a good deal of attention (Goggin et al., 1998; Klingler et al., 2001; Wang et al., 2001; Cevik & King, 2002; Pascal et al., 2002; Hartman, 2004). Aphid resistant maize and wheat lines developed by traditional plant breeding techniques have had some success in limiting aphid damage (Walter & Brunson, 1946; Auclair, 1989; Thackray et al., 1990; Quisenberry & Schotzko, 1994). However, transgenic aphid resistance would free breeders from being limited to the narrow range of germplasm that contains natural resistance genes, which would be a major advantage.

Indeed, aphid resistant transgenic plants have been developed. These plants express a plant defensive compound, the snowdrop lectin GNA (*Galanthus nivalis* agglutinin) (Hilder et al., 1995; Rahbe et al., 1995; Down et al., 1996; Gatehouse et al., 1996; Sauvion et al., 1996; Stoger et al., 1998). However, because many lectins are toxic to mammals (even though GNA is not) (Pusztai et al., 1993), this aphid resistance technology has not been adopted for commercialization. In addition, numerous transgenic plants have been produced that resist aphid-transmitted viruses by the use of virus-derived transgenes and post-
transcriptional gene silencing (Wilson, 1993; Anon, 1995; Miller et al., 1997; Koev et al., 1998; Baulcombe, 2002; Goldbach et al., 2003). Expression of one of several different plant virus genes within a plant provides resistance to the virus from which the gene was derived (Goldbach et al., 2003). However, this strategy is effective only for plant viruses with high sequence similarity to the transgene. There is clearly a need for additional tools for management of aphids and the diseases that they transmit to enhance agricultural productivity.

*Rhopalosiphum padi Virus (RhPV)*

RhPV was first isolated from the bird cherry-oat aphid, *Rhopalosiphum padi* (D’Arcy et al, 1981). The virus particles have an average diameter of 27 nm with icosahedral symmetry as seen in negatively stained preparations (Fig. 1). Virus yields were 0.3-0.5 mg/g of *R. padi*.

Figure 1. Electron micrograph of virus purified from *Rhopalosiphum padi*. Virus was stained with 2% phosphotungstic acid, pH 6.0. Bar=100 nm.
Although host range studies with RhPV have been limited, it is known to infect at least seven economically important aphid species: *R. padi, Schizaphis graminum, R. rufiabdominalis* (D'Arcy et al., 1981; Gildow & D'Arcy, 1988), *R. maidis, Metopolophium dirhodum, Diuraphis noxia* and *Sitobion avenae* (Wechmar & Rybicki, 1981; Williamson et al., 1989). Most of these aphids are vectors of barley yellow dwarf virus (BYDV) (D'Arcy et al., 1981; Gildow & D'Arcy, 1990). BYDV is the most widespread and economically important virus disease of cereals worldwide (D'Arcy & Burnett, 1995). *D. noxia* (the Russian wheat aphid), while not a BYDV vector is a serious pest, causing hundreds of millions of dollars in crop losses (Morrison & Peairs, 1998).

*Aphid transmission of RhPV*

RhPV reaches very high titers within the aphid but does not cause severe deleterious effects. Infection decreases longevity and fecundity of the aphid host (D'Arcy et al., 1981). Gildow and D'Arcy (1990) studied the tissue specificity of RhPV by inoculation of virus free clones of aphid species with density gradient-purified RhPV. Aphids (*R. padi* and *Schizaphis graminum*) were allowed to acquire virus by membrane feeding on 10 μg/ml of RhPV in sucrose. Aphids were then examined by immunoelectron microscopy (IEM). Virus was found in the midgut and frequently in the hind gut of the infected aphid. Virus was not observed in the anterior midgut, in accessory or principal salivary glands (Fig. 2). Infection resulted in progressive loss of cytoplasmic organelles, especially ribosomes, and in the development of small 200 nm membrane vesicles (Gildow and D'Arcy, 1990). Virions accumulated only in the cytoplasm and were subsequently released into the gut lumen and hemolymph.
Figure 2. Pathway of RhPV horizontal transmission. RhPV ingested on feeding is taken up by mid- and hindgut tissues by presumed receptor-mediated endocytosis (Gildow & Darcy, 1990). Following replication in these tissues, virions produced in the cytoplasm are released into the hemocoel and back into the gut. Transmission to the plant may occur either via the salivary glands, on regurgitation of virions or via the gut during probing of plant tissues. The plant acts as a virus reservoir. HG, hindgut; MG, midgut; ASG, accessory salivary gland; PSG, primary salivary gland.

An unusual feature of RhPV is its ability to utilize plants as reservoirs (Gildow, 1988). Plants can play a role in horizontal RhPV transmission between individual aphids of the same or different species. RhPV does not persist in plants (barley) indefinitely, but has been detected at low levels three weeks after contamination in the absence of aphids (Gildow & D'Arcy, 1988; Williamson et al., 1989). RhPV is only the second known example of a plant transmitted insect virus. The leafhopper A reovirus from Cicadulina bimaculata also uses plants (maize) as passive reservoirs (Ofori & Francki, 1985). The details of factors that mediate virus recognition and uptake into the gut cells are unknown. It is also unknown whether RhPV is transmitted to plants via the aphid gut, or via the salivary glands, although virus was not detected in the salivary glands in a previous study (Gildow and D'Arcy, 1998).
Genome organization of RhPV

RhPV belongs to the newly-defined *Dicistroviridae* family (ICTV, 2004). This emerging virus group includes the *Drosophila C* virus (DCV), *Plautia stali* intestine virus (PSIV) (Sasaki et al., 1998), cricket paralysis virus (CrPV), and many others. The dicistroviruses seem to be biologically isolated from vertebrate hosts because viruses with similar genome organization have not been isolated from vertebrates. These viruses are however, related to picornaviruses in protein sequence and structure, but differ in genome organization, translation control signals, and in the order of capsid protein sequences within the genome (Tate et al., 1999) (Fig. 3).

![Figure 3. Genome organization of RhPV. The 10,011 nt RNA genome of RhPV encodes two polyproteins (gray boxes) with significant similarity to polyproteins of como- and picornaviruses. ORF 1 encodes the nonstructural proteins: RNA helicase (hel), cysteine protease (prot), and RNA-dependent RNA polymerase (pol). ORF2 encodes the four structural proteins, VPl through VP4. Protein VP4 (8 kDa) is not always detected. Molecular weights of the three proteins (VPl, VP2, VP3) that comprise each virion face (capsomere) are shown (K). The distinct IRESes located in the 5' UTR and intergenic region (IGR), are indicated.](image)

Like picornaviruses, dicistroviral genes are expressed from large polyproteins that are cleaved into functional proteins at specific sites by host and viral proteases (Liljas et al., 2002). Picornaviruses have just one large open reading frame (ORF) with the structural proteins encoded at the 5' end of the ORF, whereas dicistroviruses have two ORFs, with the
structural proteins encoded in a separate, 3′-proximal ORF (Fig. 3). Proteins from both ORFs are translated via internal initiation of ribosomes directly on the genomic RNA at internal ribosome entry sites (IRES) (Wilson et al., 2000b). Structural proteins accumulate in vast excess of nonstructural proteins in infected cells (Wilson et al., 2000b). The viral RNA has a genome-linked protein at the 5′ end instead of a normal cap structure, and the IRESes allow for very efficient cap-independent translation of each ORF. IRESes from picornaviruses and other RNAs have been well-studied (Hellen & Sarnow, 2001). The 5′ IRES of distroviruses needed for translation of ORF 1 and the intergenic region (IGR) IRES that facilitates translation of ORF 2 are unrelated to each other and to any other known IRESes. The 5′ IRES is at most 200 nt long, functions in cells from all kingdoms, but is still structurally ill-defined (Woolaway et al., 2001). In contrast, the IGR IRES was rapidly characterized as it provides a remarkable new way for ribosomes to enter an mRNA (Domier et al., 2000; Wilson et al., 2000b; Domier & McCoppin, 2003). The IGR IRES forms a complex pseudoknotted structure that mimics a tRNA base paired to the mRNA in the ribosomal P site. This causes the entering ribosome to immediately begin the elongation process of translation on ORF 2, and omit the highly regulated, complex process of initiation of translation (Wilson et al., 2000a). Thus, there is no AUG- or any other start codon. This process allows the virus to bypass host antiviral translation regulatory mechanisms. However, there is no evidence for the production of subgenomic RNA by these insect viruses.
**Other Dicistroviruses**

<table>
<thead>
<tr>
<th>Dicistrovirus$^{(1)}$</th>
<th>Abbrev</th>
<th>Hosts$^{(2)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cricket paralysis virus</em></td>
<td>CrPV</td>
<td><em>Teleogryllus</em> spp(O), <em>Acheta domestica</em> (O), <em>Antheraea eucalypti</em> (L), <em>Drosophila</em> spp.(D), Honey bee (Y)</td>
</tr>
<tr>
<td><em>Acute bee paralysis virus</em></td>
<td>ABPV</td>
<td>Honey bee (Y)</td>
</tr>
<tr>
<td><em>Rhopalosiphum padi virus</em></td>
<td>RhPV</td>
<td>Aphid spp. (H)</td>
</tr>
<tr>
<td><em>Black queen cell virus</em></td>
<td>BQCV</td>
<td>Honey bee (Y)</td>
</tr>
<tr>
<td><em>Drosophila C virus</em></td>
<td>DCV</td>
<td><em>Drosophila</em> spp. (D)</td>
</tr>
<tr>
<td><em>Plautia stali intestine virus</em></td>
<td>PSIV</td>
<td><em>Plautia stali</em> (H)</td>
</tr>
<tr>
<td><em>Triatoma virus</em></td>
<td>TrV</td>
<td>Triatomine bugs (H)</td>
</tr>
<tr>
<td><em>Himetobi P virus</em></td>
<td>HiPV</td>
<td>Leafhopper spp. (H)</td>
</tr>
<tr>
<td><em>Taura syndrome virus</em></td>
<td>TSV</td>
<td>Shrimp</td>
</tr>
<tr>
<td><em>Aphid lethal paralysis virus</em></td>
<td>ALPV</td>
<td>Aphid spp. (H)</td>
</tr>
</tbody>
</table>

Table 1. Names of recognized viruses are shown in italics, and tentative species in the family are shown unitalicized. Host species are shown by their common or scientific names (2) with insect host order shown in brackets (O=Orthoptera, L=Lepidoptera, Y=Hymenoptera, D=Diptera, H=Hemiptera). Only a few of the 22 reported host species for CrPV are shown
Figure 4. Consensus phenogram of virus families. Taken from Christian et al, 2003.

The above consensus phenogram summarizes the relationships within *Dicistroviridae* and their relatedness to other virus families. Within the *Dicistroviridae* there are several distinct clades. The first of these comprises CrPV, DCV, ALPV and RhPV, with ABPV probably forming sister group to this group. The other major clade comprises PSIV, HiPV, TrV and BQCV (Fig. 4). TSV is quite distinct from the other dicistroviruses and forms a separate clade. In CrPV, ABPV, BQCV, DCV, PSIV and RhPV there is an additional small capsid protein of around 8 kDa.
The CrPV/DCV Complex

Until 1997, the best characterized of the dicistrovirus was CrPV, originally isolated from the orthopterans *Teleogryllus commodus* and *T. oceanicus* in Australia, and also from the dipteran *Drosophila melanogaster*. Young crickets became paralyzed and died. The disease, which spread rapidly and killed about 95% of the colony, was shown to be caused by CrPV (Reinganum et al, 1970). The host range of CrPV is extensive and covers over 40 species that have been tested from five orders of insects. CrPV replicates in most, but not all *D. melanogaster* cell lines, causing a distinct cytopathic effect (CPE), which normally leads to cell lysis (Scotti, 1976). CrPV grow to a very high multiplicity (in excess of $10^9$ PFU/ml) in *D. melanogaster* cell culture. No cultured cell lines have been found to support the replication of RhPV and therefore detailed studies of the replication strategies have not been carried out.

DCV was isolated from laboratory populations of *D. melanogaster* in the late 1960s. DCV has subsequently been identified in a number of species of *Drosophila*. In insect hosts, both CrPV and DCV have frequently been found as inapparent infections, detected only by bioassay or serological tests. DCV has been observed to produce CPE only in *D. melanogaster* cells. In these cells, DCV causes cell clumping and cell detachment and in some instances results in cell lysis (Christian, 1992). DCV has also been detected as a persistent infection in several *D. melanogaster* cell lines. The fact that CrPV and DCV replicate in cultured cell lines has allowed their replicative strategies to be studied quite extensively. In *D. melanogaster* cells, CrPV shuts down virtually all host cell protein synthesis within several hours after infection. This contrasts sharply with DCV which shows
less ability to shut down host cell functions (Christian, 1992). The fact that CrPV eventually results in extensive CPE whereas DCV does not may be a reflection of this ability. The processing of precursor polypeptides is primarily by virus encoded protease(s) but in vitro data suggested that *Drosophila* proteases may also be involved. Coat proteins are produced in supramolar excess compared with the nonstructural proteins, supporting the model for independent initiation of the two ORFs.

**Replication of dicistroviruses in cell lines**

Continuously cultured insect cell lines have been available for some years and this has simplified the task of testing cells of different origins for their ability to support virus replication in tissue culture systems. However, differences may exist between the cultured cells and the cells *in situ* in the insect and the nature of the tissues from which the cultured cells are derived. Several insect cell lines have been tested for their ability to support replication of CrPV or DCV. Reinganum (1975) inoculated *Aedes aegypti* cells and the *Antheraea eucalypti* lepidopteran cell line with CrPV and assayed supernatant fluids from the culture flasks in early instar cricket nymphs. He detected a small increase in virus titer after four successive passages in *A. aegypti* cells but no detectable replication in *A. eucalypti* cultures. The infected *A. aegypti* cells showed no distinct CPE although slight changes in appearance were observed. After CrPV and DCV were found to be serologically related (Reinganum and Scotti, 1976) it was thought that CrPV might replicate more efficiently in *Drosophila* cells. Schneider’s DL1 cells (Schneider, 1972) were inoculated with CrPV and the results clearly demonstrated that the virus multiplied well in this cell line and that the newly synthesized virus was indistinguishable from the original inoculum in sedimentation,
density and antigenicity (Scotti, 1976). Titration of *G. mellonella* larvae showed that virus in the infected cell medium had increased over 2000-fold by 24 hours after infection.

CrPV caused a distinct CPE; infected cells became rounded several hours after inoculation and eventually disintegrated, with a standard 25 cm$^2$ culture flask of cells typically yielding $1 \times 10^9$ infectious units of virus. CrPV did not replicate in *Spodoptera frugiperda* cells but replicated in an *A. albopictus* line increasing slightly in titer but producing no obvious CPE. Although CrPV and DCV are related, their behaviour in cells differs. DL1 cells appear to support the growth of DCV but without the distinctive CPE observed following infection with CrPV, although infected cells do clump and detach from the surface of the culture flask several days after inoculation. However, CrPV but not DCV replicated in the lepidopteran line, *Lymnaaria dispers* SCL.d135 (Quiot, 1976)

Cell lines that support replication of other dicistroviruses are unknown. Although RhPV has been studied, there are no cell lines known that support replication of the virus. Despite several attempts by several investigators (Hirumi and Maramorosch, 1971; Peters and Black, 1970) continuous cell lines derived from aphids have not been established (Christian and Scotti, 1998). Chapter 2 describes the screening of dipteran, homopteran, and lepidopteran cell lines for susceptibility to RhPV infection.

**Baculovirus expression of RhPV**

In Chapter 3 we describe baculovirus expression of RhPV. Baculovirus expression of RhPV provides an alternative approach for *in vitro* production of RhPV. This approach was adopted in case a cell line that supports replication of RhPV was not identified. The RhPV genome was cloned into a baculovirus expression vector. Baculovirus-infected cells were
examined for expression of viral proteins and assembly of virus-like particles (VLPs). VLPs were then tested for infectivity to aphids.

**Production of virus-like particles using the baculovirus expression system**

The baculovirus expression system has been used for production of a wide range of virus-like particles (VLPs) (Casal, 2001; Maranga et al., 2002) including four members of two plant virus families, namely the Comoviridae - *Arabis mosaic* (Bertioli et al., 1991), *Tobacco ringspot* (Singh et al., 1995), and *Cowpea mosaic virus* (Shanks and Lomonossoff, 2000); and the Luteoviridae - *Beet western yellows virus* (BWYV) (Tian et al., 1995) and *Potato leafroll virus* (PLRV) (Gildow et al., 2000; Lamb et al., 1996). For BWYV, Tian *et al* (1995) demonstrated production of VLPs in insect cells, but were unable to purify the VLPs. Simultaneous expression of four bluetongue virus proteins by recombinant baculoviruses resulted in correct assembly of virus-like particles (Belyaev and Roy, 1993; Hewat *et al*., 1994). Production of VLPs results from the ability of one or more capsid proteins of a virus to self-assemble into multimeric structures that are morphologically and structurally identical to the original virus. A variety of different strategies are currently under investigation in order to utilize the baculovirus insect cell expression system for efficient display on the surface of virus particles as well as on the surface of virally infected insect cells. Increasing the transfection efficiency, optimizing cloning procedures, and establishing applicable selection methods have lead to the development of a powerful tool for drug screening and ligand screening.
Figure 5. Generation of recombinant baculoviruses and gene expression with the BAC-TO-BAC Expression System. The RhPV genome was cloned into the pFASTBAC donor plasmid, and the recombinant plasmid was transformed into DH10BAC competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFASTBAC donor plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids were identified by the disruption of the lacZα gene. High molecular weight mini-prep DNA was prepared from the selected E. coli clones that contained the recombinant bacmid, and this DNA was then used to transfect insect cells.

**BAC-TO-BAC Baculovirus Expression System**

Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. Heterologous genes placed under the transcriptional control of a strong polyhedron promoter of the Autographa californica nucleopolyhedrovirus (AcNPV) are often abundantly expressed during late stages
of infection. In most cases recombinant proteins are processed, modified, and targeted to their appropriate locations, where they are functionally similar to their authentic counterparts (Luckow, 1991; O’Reilly et al., 1992; King and Possee, 1992; Luckow, 1993).

A rapid and efficient method to generate recombinant baculoviruses based on site-specific transposition of an expression cassette into baculovirus shuttle vector (bacmid) propagated in *E. coli* was developed (Luckow et al., 1993) (Fig. 5). Genes to be expressed are inserted into the multiple cloning site of a pFASTBAC donor plasmid downstream from the baculovirus-specific promoter. The plasmid pFASTBAC 1 (Anderson et al., 1996) is used to generate viruses which express unfused recombinant proteins. The bacmid contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lacZa* peptide. Inserted into the N-terminus of the *lacZa* gene, is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-attTn7) that does not disrupt the reading frame of the *lacZa* peptide. The bacmid propagates in *Escherichia coli* DH10BAC as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (*Lac*⁺) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG.

Recombinant bacmids are constructed by transposing the mini-Tn7 element from a pFASTBAC donor plasmid to the mini-attTn7 attachment site on the bacmid when the Tn7 transposition functions are provided *in trans* by a helper plasmid (pMON7124). The helper plasmid confers resistance to the tetracycline and encodes transposase. Recombinant bacmid DNA can be rapidly isolated from small scale cultures and then used to transfect insect cells (Sf9). Viral stocks (>10⁷ pfu/ml) harvested from the transfected cells can then be used to
infect fresh insect cells (Sf9 or Sf21) for subsequent protein expression, purification, and analysis.

**Acquire an infectious clone of RhPV**

The genome of positive-strand RNA viruses functions as mRNA from which all viral proteins necessary for virus propagation are translated. Thus, genomic RNA, as well as RNA transcripts from full-length cDNA clones, should be infectious. The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as reverse genetics (Pekosz et al., 1999). Such reverse genetics systems have been developed for a number of positive-stranded RNA viruses, including picornaviruses, caliciviruses, alphaviruses, flaviviruses and arteriviruses, whose RNA genomes range in size from ~7 to 15 kb in length (Yount et al., 2000). The production of cDNA clones and/or PCR-amplicons, from which infectious RNA can be transcribed *in vitro*, is an essential step in the development of reverse genetics systems for these viruses. The availability of these clones/PCR-amplicons has facilitated the study of the genetic expression and replication of RNA viruses by the use of mutagenesis, deletions and insertions and by complementation experiments. It has also enhanced the understanding of the molecular mechanisms of natural or induced RNA recombination and of plant–virus interactions such as cell-to-cell movement. This has resulted in the development of new virus vectors and vaccines (Boyer & Haenni, 1994).

The production of infectious RNA transcripts from PCR-amplicons has become a method of choice for many investigators because of the improvements in PCR in terms of fidelity and length of amplification. The pioneering work of Gritsun and Gould (1995, 1998) has also resulted in the improvement of this method by using a combination of primer sets...
and optimizing their concentrations. This method is simple, rapid and it overcomes the problem of the instability of certain sequences in bacteria (Hayes & Buck, 1990; Gritsun & Gould, 1995, 1998; Tellier et al., 1996; Campbell & Pletnev, 2000). In Chapter 4, production of infectious transcripts of RhPV is described and this is achieved by using the cell lines susceptible for RhPV infection (Z10-2 and DMII).

To our knowledge, the only dicistrovirus for which a full-length infectious transcript has been constructed is black queen cell virus (BQCV) (Benjeddou et al., 2002), by the use of long RT-PCR. However, this transcript was transcribed directly from uncloned RT-PCR product. In Chapter 4, I describe construction of a cloned, full-length infectious genome of RhPV.

**Dissertation Organization**

Our long term goal is to produce transgenic crop plants that are resistant to aphids by using RhPV as a delivery system for insect toxins that are active in the aphid hemocoel. The objective of this application is to determine the feasibility of producing transgenic plants that express RhPV, to deliver insect toxins into the hemolymph of aphids. The specific goals of my project are (i) to identify an established cell line that supports replication of RhPV, (ii) to construct an infectious clone of the RhPV genome.

Chapters 2, 3 and 4 contain manuscripts to be published. Chapter 2 is a manuscript to be submitted to the Journal of Invertebrate Pathology. This presents data showing the susceptibility of RhPV in the homopteran cell lines namely Z10-2 and DMII. Chapter 3 is a manuscript to be submitted to Journal of General Virology. This chapter describes production of RhPV virus-like particles by using baculovirus expression system. The full length cDNA clone of RhPV was constructed in pFASTBAC1 expression vector by
assembling three RT-PCR fragments. Randy C. Beckett, co-author of this manuscript has contributed by cloning the second RT-PCR fragment into the vector. Chapter 4 is a manuscript to be submitted to Journal of Virology. This chapter describes construction and testing of an infectious clone of RhPV. Chapter 5 consists of general conclusions and discussion of the project as a whole.
CHAPTER 2

IDENTIFICATION OF INSECT CELL LINES SUSCEPTIBLE TO RHOPALOSIPHUM PADI VIRUS (DICISTROVIRIDAE)

Sandhya Boyapalle,1 W. A. Miller,2 Bryony C. Bonning,1

1 Departments of Entomology and 2 Plant Pathology, Iowa State University, Ames, IA-50011

Paper to be submitted to Journal of Invertebrate Pathology

Abstract

Rhopalosiphum padi virus (RhPV) (Dicistroviridae; Cripavirus) is an icosahedral aphid virus with a 10 kb positive-sense RNA genome. It has not been propagated in cell culture because no cell lines are known to support RhPV replication. For further study of the biology of RhPV, finding a cell culture system that allows replication of the virus is essential. We screened lepidopteran, dipteran and homopteran cell lines (9 in total) for susceptibility to RhPV following RNA transfection. We observed cytopathic effects (CPE) only in cell lines derived from homopterans, the glassy winged sharp shooter, Homalodisca coagulata (Kamita et al., in press) and the corn leaf hopper, Dalbulus maidis (McIntosh, unpublished data). Infection, viral replication and production of virions was confirmed by northern blot hybridization, RT-PCR, western blot analysis and immunoelectron microscopy.

Introduction

RhPV was first isolated from the bird cherry-oat aphid, Rhopalosiphum padi, which is a pest of Gramineae, especially maize, barley, oats and wheat. Although host range studies with RhPV have been limited, it is known to infect at least seven economically important aphid species: R. padi, Schizaphis graminum, R. rufigibberosus (D'Arcy, Burnett, and Hewings, 1981; Gildow and J., 1988), R. maidis, Metopolophium dirhodum, Diuraphis noxia
and *Sitobion avenae* (Wechmar and Rybicki, 1981; Williamson, Wechmar, and Rybicki, 1989). RhPV belongs to the newly-defined *Dicistroviridae* family (ICTV, 2004). The dicistroviruses are characterized by host ranges restricted to invertebrates and a positive sense RNA genome with a characteristic dicistronic arrangement of the genome (Christian et al., 1998). Other members of the family include *Drosophila* C virus (DCV, Johnson and Christian, 1998), *Plautia stali intestine virus* (PSIV, (Sasaki et al., 1998), Himetobi P virus (HiPV, Nakashima et al., 1999) cricket paralysis virus (CrPV, Reinganum et al., 1970), acute bee paralysis virus (ABPV, Govan et al., 2000), black queen cell virus (BQCV, Leat et al., 2000).

The positive-stranded RNA genomes of dicistroviruses contain two open reading frames (ORF). The 5' ORF encodes a polyprotein that includes all nonstructural proteins including RdRp, while the second one codes for the structural polyprotein. Proteins from both ORFs are translated via internal initiation of ribosomes directly on the genomic RNA at internal ribosome entry sites (IRES) (Wilson et al., 2000). Activity of 5' IRES has been demonstrated in mammalian, *Drosophila*, and wheat germ *in vitro* translation systems, and also in *Spodoptera frugiperda* (Sf21) cells, used in baculovirus expression system (Royall et al., 2004). Structural proteins accumulate in vast excess of nonstructural proteins in infected cells (Wilson et al., 2000).

CrPV has a wide host range and is widely distributed in nature. CrPV has been isolated from members of the *Orthoptera, Hymenoptera, Lepidoptera, Hemiptera,* and *Diptera*. DCV has been isolated only from dipteran species while HiPV, PSIV, and RhPV have only been isolated from hemipteran species. CrPV and DCV replicate readily in several *Drosophila* cell lines. CrPV also replicates in a number of other insect cell lines (Scotti et al,
1996; Christian and Scotti, 1998). Cell lines that support replication of other dicistroviruses are unknown. Although RhPV has been studied, there are no cell lines known that support replication of the virus. Despite several attempts by several investigators (Hirumi and Maramorosch, 1971; Peters and Black, 1970) continuous cell lines derived from aphids have not been established (Christian and Scotti, 1998). Cells cultured from the *Achrythosiphum pisum* (Hirumi and Maramorosch, 1971) and *Hyperomyzus lactucae* (Peters and Black, 1970) were viable only for a short time (Boyapalle, unpublished observation).

For further study of the biology of RhPV, finding a cell culture system that allows replication of the virus is essential. Here, nine cell lines including lines derived from Lepidoptera, Diptera and Homoptera were tested for susceptibility to infection by RhPV. We identified two homopteran cell lines that support replication and packaging of RhPV.

**Methods**

**Viruses and cells.** RhPV was purified from infected colonies of *R. padi* maintained at Iowa State University, as previously described by D’Arcy et al. (1981). CrPV and DCV were propagated in cultured *Drosophila melanogaster* cells (Schneider 2: S2). The viruses were purified from infected S2 cells as described by Krishna et al. (2003).

Sf21 cells also derived from *Spodoptera frugiperda* (Vaughn et al., 1977) were maintained in TC-100 insect cell medium (Sigma Chemicals, St Louis, Missouri, U.S.A) supplemented with 10% FBS. Sf9 cells derived from Sf21 cells were maintained in Hink’s TNM (JRH Biosciences) supplemented with 3% fetal bovine serum (FBS; Gibco-BRL)). Tn5B1-4 “High-Five” cells derived from the cabbage moth *Trichoplusia ni* (Wickham et al., 1992) were maintained in Excel 405 (JRH Biosciences) serum free medium. OnE cells derived from *Ostrinia nubialis* were maintained in Excel 405 (JRH Biosciences)
supplemented with 3% FBS. S2 cells derived from fruit fly *D. melanogaster* (Schneider, 1972) were maintained in Schneider’s media (Sigma Chemicals, St Louis, Missouri, U.S.A.) supplemented with 10% FBS. Cell lines derived from *Agallia constricta* (AC20) (McIntosh et al., 1973), *Anticarcia gemmatalis* (BCIRL-AG-AM) (McIntosh and Ignoffo, 1989), corn leaf hopper *Dalbulus maidis* (DMII) (A. McIntosh, University of Missouri, Columbia) glassy winged sharp shooter *Homalodisca coagulata* (Z10-2) (Kamita et al., in press) were maintained in Excel 405 (JRH Biosciences), supplemented with 10% FBS. The media supporting the above cell lines was supplemented with 1% penicillin-streptomycin (Sigma) except Schneider’s medium which had 1% penicillin-streptomycin-amphotericin B (Invitrogen)

**Isolation and transfection of viral RNA.** Viral RNA was extracted using Absolutely RNA® RT-PCR miniprep kit (Stratagene) according to the manufacturer’s specifications. The RNA concentration was determined by measurement of absorbance at 260 nm. To test the ability of the cell lines to support replication of RhPV, DCV, and CrPV, the following procedure was employed (Masoumi et al, 2003). Each cell line was seeded into four wells of a 6 well plate (Fisher Scientific). Cell density per well was 2x10⁵ for Sf9 and AC20 cells; 1x10⁵ for Sf21, Hi-5, OnE and AGAM cells; 6x10⁵ for S2 cells. Z10-2 and DMII cells were near confluent in each well. The cells were allowed to attach to the surface for 4 h at room temperature. Media and floating cells were removed and the cells washed with FBS free medium. One well was treated with 0.5 μg DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N, N-trimethylammonium methyl-sulfate (Alexis) in 1 ml of FBS-free medium. The second, third and fourth wells were treated with 0.5 μg DOTAP in 1 ml FBS-free medium along with 4 μg of RhPV RNA, 2 μg DCV RNA and CrPV RNA respectively. All treatments were left
at 28°C for 6 h, cells were gently washed twice with FBS containing media except Hi-5 cells which were washed in FBS free medium. The cells were then incubated in 1.5 ml of their respective media at 28°C. Cells were examined daily for CPE and harvested after 4-8 days.

**Western blot analysis of proteins.** Harvested cells were washed twice in phosphate-buffered saline (PBS) and resuspended in 100 µl of PBS. Portions were boiled for 5 min in 100 µl of 2x protein dissociation buffer (2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM-Tris-HCl, 0.01% bromophenol blue, pH 6.8) and resolved by SDS-PAGE. Approximately 150 ng of purified virus alone was run as a positive control in each gel. Ten micro liter samples were resolved by electrophoresis on 4-12% SDS-PAGE gels (Bio-Rad Laboratories), after which each gel was treated for 30 min with Towbin buffer (10 mM Tris base, 96 mM glycine in 10% methanol). The proteins were transferred to Hybond-P membrane (Amersham Pharmacia) in Towbin buffer using an electro blotter (Bio-Rad apparatus) for 1 h at 100V. The membrane was placed for 2 h at room temperature in a blocking solution consisting of 5% skimmed milk and 0.1% Tween-20 in Tris buffered saline (TBS). Rabbit polyclonal anti-RhPV antiserum was purified as described by Bassham and Raikhel (1998), diluted 100-fold in blocking solution, and incubated with the membrane for 1 h at room temperature. Polyclonal anti-DCV and anti-CrPV antisera (Christian and Scotti, 1994) were diluted 2000-fold in blocking solution, and incubated with the membrane for 1 h at room temperature. Membranes were washed twice with 0.1% Tween-20 in TBS before reaction with goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Sigma). After washing for 1 h, the bound enzyme was detected using the ECL detection system (Amersham Pharmacia).
**Plasmids.** Full length double stranded cDNA of RhPV was cloned between the *EcoRI-KpnI* sites of pGEM-3ZF (Promega) (unpublished data). The T7 promoter was positioned upstream of 5' end of the RhPV sequence and SP6 downstream of the 3' end of RhPV sequence in the vector. The RNA synthesized by T7 RNA polymerase is complementary to the RNA synthesized by SP6 RNA polymerase.

**Preparation of in vitro transcripts.** The above plasmid was linearized with *Acc651*, and used as template for *in vitro* transcription of full length uncapped RNA from the T7 promoter at the 5' end using T7 MegaScript kit (Ambion). Full length negative sense RNA was acquired by linearizing the plasmid with *EcoRI*, and used as template for *in vitro* transcription from the SP6 promoter using SP6 Megascript kit (Ambion). Strand specific ^32^P labeled RNA probes were prepared from the T7 and SP6 promoters present in the vector after the plasmid was digested with *HpaI*.

**Northern blot hybridization.** To analyze the production of RNA over the course of infection, Z10-2 cells were transfected with RhPV RNA and full length negative sense RhPV transcript as described above. Transfected wells were harvested at time points 0, 0.2, 12, 24, 48, 72, 96 hours post transfection (hpt) and total cellular RNAs were extracted using Trizol (Invitrogen) according to the manufacturer's specifications. RNA was analyzed by Northern blot as described by Koev et al. (1999). Approximately 100 ng of the viral RNA spiked with total RNA from mock infected cells or full length negative sense RhPV transcript was run along side as positive control. A ^32^P-labeled probe complementary to the 1.9-kb 3'-terminal sequence of RhPV (plasmid digested with *HpaI* and transcribed using SP6 RNA polymerase) was used to detect production of positive strand RNA in the cells. A ^32^P-labeled probe complementary to the 1.2-kb 5' end sequence of RhPV (plasmid digested with *HpaI* and
transcribed using T7 RNA polymerase) was used to detect negative sense RhPV RNA produced in the cells.

**Purification of virus particles from the infected Z10-2 cells.** Z10-2 cells were harvested when extensive CPE were observed (typically at 4 dpt) and the virus particles were purified as described by Krishna et al (2003). Purified virus particles were negatively stained with 2% uranyl acetate and examined by TEM.

**Immunogold electron microscopy.** Z10-2 cells and DMII cells were grown to near confluence in T-25 flasks (Fisher Scientific), two for each cell line. The cells were transfected as described above with 1µg of DOTAP and 10 µg of RhPV RNA. The cells were harvested 4 dpt (Z10-2) cells and 10 dpt (DMII cells). The cells were pelleted and fixed in 1% paraformaldehyde-0.5% gluteraldehyde-0.05% sodium cacodylate, pH 7.1, for 10 min at 4°C, then in 2% paraformaldehyde-2.5% gluteraldehyde-0.05% sodium cacodylate, pH7.1, for 30 min at 4°C. After washing the cells three times (10 min each time) with 0.05M sodium cacodylate, the cells were dehydrated with a series of ethanol concentrations (50%, 70%, 85%, 95%, 3X100%) for 30 min at each step at 4°C. Cells were then infiltrated with ethanol: LR White Resin using ratios of 1:1 (2 h at 4°C), and 1:3 (overnight at 4°C) followed by pure LR White (24 h at 4°C) changing resin every 12 h. The cells were later embedded in gelatin capsules, and resin was polymerized at 4°C for 48 h with the UV light on.

Gold labeling of the grids was carried out as described by Erickson (1993). Grids were treated with 25 µl of TBS-supplemented buffer (0.05M Tris, 0.85% NaCl, pH 8.3-8.5, 0.5% normal goat serum, 0.5% normal pig serum and 0.5% BSA) with 3% non-fat dry milk for 2 h at room temperature. Grids were then immersed in 50 µl drops of 1:10 purified rabbit polyclonal anti-RhPV antiserum diluted in TBS-supplemented buffer with 3% non-fat dry
milk for 5 h at 37°C. After washing the grids three times in TBS-supplemented buffer, the grids were treated with 25 μl drops of 1:100 dilution of secondary goat anti-rabbit antibody conjugated with 10 nm gold particles (Ted Pella Inc.) for 1 h at room temperature. After stream washing and three washes in drops of distilled water (10 min each wash), grids were dried and stained with 2% aqueous uranyl acetate for 5 min and examined on a JOEL 1200 EX scanning/transmission electron microscope at 80 KV.

Results

CrPV and DCV infection of cultured cell lines

The ability of RhPV to replicate in various insect cell lines was tested by transfecting the cells with viral RNA (Fig. 1). DCV and CrPV RNAs were used as positive controls for RhPV transfection experiments. A summary of the transfection results is presented in Table 1. The Drosophila (S2) cell line was permissive to CrPV and DCV as described previously, and exhibited distinct CPE 2-3 dpt (Reinganum, 1975; Plus et al., 1975, 1978). By day 5 dpt there were no living cells in either of the transfections in S2 cells. The cells treated with DOTAP alone (negative control) were healthy at 5 dpt and continued to divide. In the case of A. gemmatalis the transfected cells showed signs of swelling/hypotrophy, with some evidence of cell lysis and blebbing. The cells detached from the surface (Masoumi et al, 2003). CPE were more pronounced in CrPV-transfected cells (3-4 dpt compared to those transfected with DCV RNA (5 dpt).

For the Z10-2 cell line, cells showed signs of CPE by 3-4 days for both CrPV and DCV. The cells lost their characteristic fibroblast-like extensions, became rounded and were of uniform size. They detached from the substrate with approximately 90% of the cells floating. On examination with a phase contrast microscope, the cells had a granular
appearance with some evidence of cell lysis. In contrast, cells in the control wells were dividing and healthy with characteristic fibroblast-like extensions. There were no granular inclusions in the cytoplasm of the control cells. There were only a few floating cells in the control wells, which in contrast to the infected cells, varied in size and shape.

For the DMII cell line, the cells transfected with CrPV and DCV RNA exhibited CPE in 4-6 dpt. The cells were rounded and looked strikingly different from cells in the control wells. Infected cells lost their surface extensions, but did not float. The cells were clumped and the cytoplasm appeared granular. None of the other five cell lines tested were permissive for CrPV or DCV replication by viral RNA transfection.

**Ability of RhPV to replicate in cultured cells.**

As for CrPV and DCV viral RNA transfections, RhPV RNA transfections were carried out for all cell lines listed in Table 1. Only Z10-2 and DMII cell lines were permissive to infection by RhPV RNA. CPE were observed in Z10-2 cells at 4 dpt (Fig 2B), while the control cells appeared healthy, with fibroblast-like extensions (Fig. 2A). DMII cells showed CPE 8-10 dpt that were similar to those described above (Fig 2D and E). To determine whether CPE could be caused by infectious viral RNA, transfections were carried on Z10-2 cells with the *in vitro* synthesized full length negative sense RhPV RNA transcript. There was no difference between these cells and control cells (treated with DOTAP only) at 4 dpt (Fig 2A and 2C). This result shows that the CPE observed resulted from the infectious viral RNA.

**Detection of coat proteins following viral RNA transfections.**

Western blot analysis was used to determine whether transfection of cells with viral RNA resulted in synthesis of viral coat proteins (Fig. 3B). Purified viral protein samples of
RhPV, DCV and CrPV were analyzed by SDS-PAGE (Fig. 3A) with reference to molecular weight markers. RhPV has three major proteins of molecular weights 30, 29 and 28 kDa (Williamson et al., 1988). The fourth coat protein (VP4) has not been detected by Western blot. Gildow and D’Arcy (1990) implicated that VP4 can be detected in protein gels. The capsid proteins for DCV are 37.7, 33.3 and 29.2 kDa (Jousset et al., 1977), and for CrPV are 35, 34, and 30 kDa (Moore et al., 1985). Western blot analysis was carried out using polyclonal antibodies raised in rabbits against purified RhPV, DCV or CrPV. Antibody for RhPV was affinity purified using the viral coat proteins alone (Bassham and Raikhel, 1998) (Fig 3B). CPE resulting from viral RNA transfection in some cell lines correlated with accumulation of viral structural proteins as determined by western blot analysis (Table 1).

**Synthesis of viral RNA following viral RNA transfection**

We examined viral RNA accumulation in Z10-2 cells transfected with RhPV RNA. Northern blot hybridization was used to detect accumulation of positive and negative sense genomic RNA molecules (Fig. 4A and 4C). Input positive-sense viral RNA and negative-sense *in vitro* transcript were detected at 12 and 24 hpt (Fig 4A and 4B). Blots revealed that substantial amounts of positive sense viral RNA were present at least up to 96 hpt (Fig. 4A). While a striking increase is not apparent, the levels must be compared against the large amount of inoculum present at early time points, much of which degraded by 12 hpt (low molecular weight smear). For comparison, non-replicatable RNA (negative sense inoculum) levels decreased rapidly after 24 hr, (Fig. 4B).

To confirm replication of the RhPV RNA, we tried to detect negative strand RhPV RNA in cells inoculated with infectious viral RNA. Negative strand RNA was indeed detected in cells inoculated with RhPV RNA as in Fig. 4A, beyond 96 hpt, (Fig. 4C). The
negative sense RNA was not detected until 48 hpt. Between 48 and 96 hpt these negative sense RNAs appeared to have reached peak levels. Bands are not well defined because the negative strand peaks at a many fold lower concentration than that of the corresponding positive sense species (Fig 4C).

Detection of virus particles in infected cells.

To determine whether virions could be observed in the infected cells, Z10-2 cells were transfected as described above and the cells harvested at 4 dpt, when CPE were pronounced, for purification of virus particles (Krishna et al, 2003). Purified virus particles were negatively stained with 2% uranyl acetate and examined by TEM (Fig 5B). RhPV particles purified from aphids (Fig 5A) were examined for comparison. The size of the particles was 26 - 27 nm as observed by Rybicki, (1984). The virus particles purified from Z10-2 cells 4 dpt with DCV RNA were negatively stained and analyzed by TEM. The average size of the particles was 29 - 30 nm as previously described (Reinganum, 1975; Jousset et al., 1977).

Immunolocalization of RhPV in Z10-2 and DMII cells

To determine the intracellular localization of the virus particles, Z10-2 and DMII cells were transfected as described above, harvested 4 dpt and sectioned for examination by TEM. Affinity purified RhPV antiserum was used for immunolocalization of virus particles. Infected cells were compared to mock-infected Z10-2 (Fig. 6A and 6B) and DMII cells (Fig. 6C and 6D). In the infected cells, large electron-dense amorphous aggregates (up to 2μm in diameter and oval-shaped inclusions were labeled by anti-RhPV antiserum in the cytoplasm (Fig. 6A and 6C). These cytoplasmic structures appeared to be enclosed by a membrane.
Almost every DMII cell (8-10 dpt), had at least one virus-induced cytoplasmic structure (Fig. 6C, arrows), and most cells had numerous structures.

Moreover, viral antigen was concentrated in electron-lucid patches in the cytoplasm, probably consisting of small vesicles (Fig. 6F, arrows). Mock infected Z10-2 or DMII cells did not exhibit any of these cytoplasmic inclusions or labeling (Fig. 6B and 6D).

Figure 7 shows infection of Z10-2 cells (6-7 dpi) following treatment with supernatant harvested at 6 dpt from cells transfected with viral RNA. Characteristic CPE were observed, with rounding of the cells and detachment from the surface of the flask. Importantly, supernatant from transfected Z10-2 cells contained a substantial amount of infectious virus at 6 dpt.

**Discussion**

Here we identify two insect cell lines that support replication of RhPV. Although RhPV, CrPV and DCV are related, their behavior in the cells was quite different. CrPV and DCV caused CPE in Z10-2 and DMII cells in almost half the number of days required for RhPV to do so. Extensive cell lysis was observed with CrPV and DCV infection compared to that of RhPV, although the cells became rounded and detached from the surface with limited cell lysis at 4 dpt. RhPV required double the amounts of viral RNA inoculum compared to CrPV and DCV to exhibit similar CPE. Scotti made similar observations with CrPV and DCV; DL1 (*Drosophila melanogaster*) cells appeared to support growth of DCV but without distinctive CPE observed for CrPV infections, although the infected cells did clump and detached from the surface of the culture flask several days after inoculation (Scotti et al., 1981).
Western blot analysis summarized in Table 1 revealed that viral RNA transfected into Z10-2 and DMII cells was translated and the ORF2 polyprotein was efficiently processed into structural proteins. These data clearly demonstrate that IRES elements are active (5' IRES and IGR-IRES). Detection of structural proteins is not in itself indicative of a cell line being permissive for virus replication however. Masoumi et al (2003) observed that CrPV was unable to infect and/or replicate in either T. ni or A. aegypti even though both IRES elements were functional in these cell lines. These findings were in general agreement with the findings of Shaw- Jackson and Michiels, (1999) for Theiler’s virus that IRES elements may be active in cell types that are not from the natural hosts of the virus.

While there was no striking increase in the amount of viral RNA detected in Z10-2 cells (Fig. 4A), comparison with cells transfected with the non replicatable negative-sense RNA (Fig. 4B) indicates that viral RNA replication occurred in these cells. The large amounts of RNA detected early represents inoculum. It is unlikely that inoculum RNA would not decrease by 48 or 72 hpt. The low molecular weight smear at 12 h shows degradation of inoculum. Thus, the RNA at the later time points almost certainly arises due to replication. Consistent with this, low amounts of negative strand was detected (Fig 4C), these are fuzzy smears, presumably because (+) strand is present in vast molar excess to (-) strand. This result suggests that cellular factors required for the processing of viral gene products and packaging were available in these homopteran cells. Virus particles were purified from the infected cells, 4 dpt as described by Krishna et al (2003), and negatively stained and examined by TEM. The particles were morphologically indistinguishable from the virus particles purified from aphids. The size was determined to be 26-27 nm as reported by Rybicki (1984). DCV particles purified from the infected Z10-2 cells (4 dpt) were 29-
30 nm in size, slightly larger than the RhPV particles. DCV particles were previously estimated to be 30 nm (Jousset et al., 1977).

RhPV particles appear to localize in the cytoplasm of the infected cell (Fig. 6A and 6C). In host specificity studies of RhPV in aphids, RhPV particles occurred free in the cytoplasm and also packed in crystalline arrays in large membrane vesicles (Gildow and D’Arcy, 1990). Virions were never observed in the nucleus and the nuclear envelope and mitochondria of the gut cells remained intact following virus replication and development of severe cytopathic symptoms. The authors also observed that clusters of single membrane vesicles were associated with the virions during early stages of infection. Occasionally, a clump of label was observed associated with the cell membrane (Fig. 6E), which may indicate a site of viral endocytosis or release.

DCV and CrPV virions are also found in crystalline arrays in the cytoplasm of infected cells, as for vertebrate picornaviruses (Reinganum et al., 1970; Jousset et al., 1972; Jousset, 1975). Our EM observations (Fig. 6A and 6C) also revealed formation of electron dense amorphous cytoplasmic structures that were strongly labeled by anti-RhPV antibodies. Similar structures were observed within the cytoplasm of the stomach cells of the pea aphid infected with Acrythosiphum pisum virus (van den Huevel et al., 1997). Abundant cytoplasmic structures loaded with virions were seen when a midgut cell line derived from Helicoverpa zea was infected with Providence virus (Tetraviridae) (Pringle et al., 2003).

We have shown that two homopteran cell lines (Z10-2 and DMII) are permissive to RhPV infection, including translation of viral gene products, virus replication and packaging of the virus particles. Future research will focus on the development of an infectious clone of RhPV to facilitate studies of replication, assembly and structure of the virus.
Acknowledgements

The authors thank Dr. Art McIntosh, USDA- ARS Columbia, Missouri for providing DMII, AC-20 and AGAM cell lines, and Dr. Shizuo G. Kamita, University of California, Davis for providing Z10-2 cells. The authors would like to thank Dr. Peter Christian, National Institute for Biological Standards and Control, UK for provision of CrPV, DCV and the respective antisera. This material is based upon work supported by an Iowa State University Carver Trust grant as well as Hatch Act and State of Iowa funds.

References


Characterization of cell lines developed from glassy winged sharp shooter, *Homalodisca coagulata* (Hemiptera:Cicadellidae) (In press)


Fig1. RhPV genomic RNA. Ethidium bromide stained agarose gel. Lane1 RNA ladder (kb); Lane 2 RNA (2 μg) extracted from R. padi derived RhPV purified by sucrose gradient centrifugation.
Table 1. Susceptibility of different insect cell lines to infection with RhPV, DCV and CrPV following transfection with viral RNA.

<table>
<thead>
<tr>
<th>Order</th>
<th>Cell line</th>
<th>RhPV</th>
<th>DCV</th>
<th>CrPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CPE</td>
<td>Western</td>
<td>CPE</td>
</tr>
<tr>
<td>Lepidoptera</td>
<td><em>Spodoptera frugiperda</em> Sf9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>S. frugipera</em> Sf21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Trichoplusia ni</em> Tn5B1-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Ostrinia nubilalis</em> OnE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Anticarsia gemmatalis</em> AM1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diptera</td>
<td><em>Drosophila melanogaster</em> S2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Agallia constricta</em> AC-20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Homoptera</td>
<td><em>Homalodisca coagulata</em> Z10-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Dalbulus maidis</em> DMII-AM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

CPE-cytopathic effects
Western-Western blot analyses with the appropriate antiserum.
DCV-*Drosophila* C virus
CrPV-*Cricket paralysis* virus
Fig. 2. Transfection of Z10-2 cells with RhPV RNA (A - C). (A) Z10-2 cells treated with DOTAP alone, negative control; (B) Z10-2 cells transfected with 10μg of RhPV RNA showing CPE at 4 days post transfection (dpt). Note the rounding of the cells, and reductions in fibroblast-like extensions and cell number. 90% of the cells detached from the flask. (C) Z10-2 cells transfected with 10μg negative-sense full length *in vitro* transcript of RhPV, negative control. CPE was not observed 4 days post transfection. The characteristic fibroblast-like extensions (arrows) were clearly visible in control treatments (A and C).

Transfection of *D. maidis* (DMII) cells with RhPV RNA (D-E). (D) DMII cells treated with DOTAP alone, negative control. Note the fibroblast-like extensions; (E) DMII cells transfected with 10μg of RhPV RNA showing CPE at 8 days post transfection. Infected cells (E) became rounded (arrows), were of uniform size, and detached from the flask in clumps. Cell division was reduced.
Fig. 3A. Coat proteins of RhPV, DCV and CrPV. Sucrose gradient-purified virus particles were run on an SDS polyacrylamide gel and coat proteins visualized by Coomassie Blue staining. RhPV was purified from R. padi; DCV and CrPV were purified from D. melanogaster S2 cells.

Fig. 3B. Western blot analysis of the Z10-2 cells transfected with RhPV RNA. Lane 1 shows three coat proteins of sizes 28, 29, 30 kDa of RhPV derived from R. padi purified by sucrose gradient centrifugation. Lane 2 shows RhPV coat proteins purified from Z10-2 cells transfected with RhPV RNA (4 dpt).
Fig. 4. Northern blot analyses of RhPV RNA in Z10-2 cells. (A) Total RNA extracted at 0.25, 12, 24, 48, 72 and 96 hours after transfection of Z10-2 cells with viral RNA. Hybridization was carried out using a $^{32}$P-labeled 3' end-specific, negative-strand riboprobe. Controls are virion RNA extracted from virus particles (+); mock infected Z10-2 cells (-). (B) Total RNA from Z10-2 cells transfected with full-length, in vitro transcribed negative strand RhPV RNA. Hybridization was carried out using a $^{32}$P-labeled 5' end-specific, positive stranded riboprobe. Controls are in vitro synthesized full length negative sense RhPV6 RNA (+), in vitro synthesized full length positive sense RhPV6 RNA (-; 100 ng). (C) Detection of antisense RhPV RNA in Z10-2 cells. Total RNA was extracted at 12, 48, 96, 120 and 144 after transfection with virion RNA. Hybridization was carried out with a 5' end positive strand RNA specific riboprobe. In vitro synthesized full-length negative sense RhPV6 RNA (+; 200 ng) was used as positive control. Lower panels show ethidium bromide-stained ribosomal RNA that indicate total amount of RNA loaded.
Fig. 5. (A) *R. padi*-derived virus particles of RhPV. Virus particles were purified from *R. padi* by 10-40% sucrose gradient centrifugation and negatively stained with 2% uranyl acetate for analysis by TEM. (B) Z10-2 cell-derived virus particles of RhPV. Virus particles were purified from Z10-2 cells 4 dpt with RhPV RNA. The size of the particles in A and B is 26 - 27 nm. (C) Z10-2 cell-derived virus particles of DCV. Virus particles were purified from Z10-2 cells 4 dpt with DCV RNA. Virus particles were negatively stained and analyzed by TEM. The size of the particles is 27 - 29 nm. Bars = 200 nm.
Fig. 6. Immunogold labeling of RhPV virions in Z10-2 and DMII cells. (A), Z10-2 cells 4 dpt with 10µg RhPV RNA; (B) mock-infected Z10-2 cells; (C), DMII cells, 8 dpt with 10µg RhPV RNA. (D) Mock-infected DMII cells. Note the oval shaped electron dense inclusion bodies in the cytoplasm in A and C (arrows) (E) and (F) detail of infected Z10-2 cells 4dpt showing labeling of cell membrane (E), and electron lucid regions in the cytoplasm (F). Size bars, 200 nm.
Fig. 7. Reinfection of Z10-2 cells with supernatant from RhPV RNA transfected cells. A, Mock infected Z10-2 cells; B, Reinfection of Z10-2 monolayer with the supernatant from the 6 dpt Z10-2 cells with RhPV RNA. Cells were reinfected and the CPE of the infected cells was observed 6-7 days post infection (dpi). The cells were rounded, raised to the surface and the fibroblast-like extensions of the cells were lost (arrows).
CHAPTER 3

GENERATION OF RHOPALOSIPHUM PADI VIRUS (DICISTROVIRIDAE) VIRUS-LIKE PARTICLES USING THE BACULOVIRUS EXPRESSION SYSTEM

Sandhya Boyapalle,1 Randy C. Beckett,2 W. Allen. Miller,2 Bryony C. Bonning,1

1 Departments of Entomology and 2Plant Pathology, Iowa State University, Ames, IA-50011

Paper to be submitted to Journal of General Virology

Abstract

*Rhopalosiphum padi* virus (RhPV) is a small icosahedral insect virus in the family *Dicistroviridae* with a 10011 nt positive-sense RNA genome. A full length cDNA clone of RhPV was constructed and inserted into the baculovirus expression transfer vector, pFastBac1 derived from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). RhPV cDNA was placed under the control of the AcMNPV polyhedrin promoter. Expression of the genome in Sf21 cells resulted in formation of RhPV virus-like particles (VLPs) whose capsids are structurally and immunologically indistinguishable from the native virions. RhPV RNA was detected in VLPs recovered from infected cells by RT-PCR. RhPV VLPs were found to be infective to *Rhopalosiphum padi* in one out of two replicates in aphid infectivity experiments. Our results demonstrate that the baculovirus expression system can be used for production of RhPV in a non-host lepidopteran cell line. Assembly of the virus particles in the nucleus of baculovirus infected cells indicates that RhPV polyproteins are processed and that the virus encoded proteases may be functional in Sf21 cells.

Introduction

RhPV belongs to the newly-defined *Dicistroviridae* family (ICTV, 2004). Dicistroviruses are restricted to invertebrates and have a positive sense RNA genome with a
characteristic dicistronic arrangement (Christian et al., 1998). RhPV was first isolated from the bird cherry-oat aphid, *Rhopalosiphum padi*, a pest of plants in the Gramineae family, especially barley, oats and wheat. Limited host range studies indicate that it can infect at least seven economically important aphid species: *R. padi*, *Schizaphis graminum*, *R. rufiabdominalis* (D'Arcy, Burnett, and Hewings, 1981; Gildow, 1988), *R. maidis*, *Metopolophium dirhodum*, *Diuraphis noxia* and *Sitobion avenae* (Wechmar and Rybicki, 1981; Williamson et al., 1989).

Like picornaviruses, dicistroviral genes are expressed from large polyproteins that are cleaved into functional proteins at specific sites by host and viral proteases (Liljas et al., 2002). Picornaviruses have just one large open reading frame (ORF) with the structural proteins encoded at the 5’ end of the ORF, whereas dicistroviruses have two ORFs, with the structural proteins encoded in a separate, 3’-proximal ORF (Fig. 1). Proteins from both ORFs are translated via internal initiation of ribosomes directly on the genomic RNA at internal ribosome entry sites (IRES) (Wilson et al., 2000). The 5’ IRES of dicistroviruses needed for translation of ORF 1 and the intergenic region (IGR) IRES that facilitates translation of ORF 2 are unrelated to each other and to any other known IRESes.

Viral genes have been expressed for a variety of purposes by cloning them into vectors for expression in heterologous systems. Here we report the assembly of RhPV VLPs in insect cells that had been infected with a recombinant baculovirus containing the RhPV genome. The infectivity of baculovirus produced RhPV virus-like particles to aphids requires further investigation. Although the expression levels of RhPV were low, the VLPs were indistinguishable from native virions.
Methods

Virus and cells. *Spodoptera frugiperda* cells (line IPLB-Sf21) (Vaughn et al., 1977) were propagated in TC 100 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL). Sf9 cells were maintained in Hink’s TNM (JRH Biosciences) supplemented with 3% fetal bovine serum (FBS). Stock cultures were maintained in screw-cap plastic tubes (Falcon) with 25-cm² growth area. A polyhedrin-negative recombinant baculovirus expressing bacterial β-galactosidase, AcRP231acZ (Possee and Howard, 1987) was used as a control virus.

Construction of a full length cDNA clone of RhPV. The recombinant baculovirus, AcRhPV, which contains the cDNA of RhPV under control of the polyhedrin promoter was constructed using the Bac-to-Bac baculovirus expression system (Gibco-BRL). RhPV virions were purified from infected *R. padi* using sucrose gradient centrifugation. RhPV RNA was extracted from virions by using the Absolutely RNA® RT-PCR miniprep kit (Stratagene). For construction of RhPV cDNA fragments (Fig 2) first strand cDNA was synthesized from 1 µg viral RNA by M-MLV H- Reverse Transcriptase (Gibco-BRL) at 42°C for 1 h as per the manufacturer’s specifications. The GeneAMP XL PCR kit (PE Applied Biosystems) was also used as per the manufacturer’s specifications. To generate the first cDNA fragment (open rectangle in fig 2) we used the following pairs of primers: P1, 5’-GGGGG*GAATTCA*AGGATATAAAGAACCTATAATCCCTTCGCA-3’ (introduced EcoRI restriction site is italicized and underlined, polyhedron promoter sequence ATAT downstream of EcoRI was also introduced in the primer) and P2 (3224 bp), 5’-TCGCGAACAATAGATAGGCACT-3’ (*NruI* site is underlined). The resulting reverse transcription PCR (RT-PCR) product was cloned between EcoRI and *StuI* sites in the
multiple cloning site of the pFastBac1 baculovirus expression vector and the clone was named pclone 1. We attempted to produce the second fragment from 2149 bp (EcoRI) and 7070 bp (XbaI), but found that pclone 1 has an unpublished XbaI site at approximately 2000 bp, which prevented cloning using XbaI. So we used P1 and P4 (7070 bp), 5'-CTCTCCTGTGACGTATCTAGA-3' (XbaI underlined) to generate a 7 kb PCR product, which was cut with XbaI and we gel purified the larger (~5 kb) fragment (indicated by dark rectangle in Fig. 2) using the QIAquick gel purification kit (Qiagen). Pclone 1 was also cut with XbaI and we gel purified the larger fragment (~6775 bp), which includes pFastBac1 (4775 bp) and a 2 kb 5'- end fragment of the RhPV sequence. The two gel purified products were ligated and transformed into competent E. coli DH5α (Promega). The resulting clones were named pclone 2. The colonies were screened using restriction enzyme digestions and pclone 2 was selected for further cloning purposes. The third cDNA fragment (indicated by grey rectangle in Fig. 2) was generated with P5 (5680 bp), 5'-CCGCACGTTATAGCTGGCGACTATT-3' (NheI site underlined) and P6 (10011 bp), 5'-GGGGGGTTACCCACGAAAATGACATTACAG-3' (the introduced KpnI site is italicized and underlined). The amplified product was cloned between the NheI and KpnI sites of pclone 2, thus generating full length cDNA clones of RhPV. The resulting recombinant baculovirus transfer vectors were named pAcRhPV 1 through 12. Four full length clones selected based on restriction digestion analysis, were sequenced across the cloning junctions to confirm their orientation.

**Generation of recombinant baculoviruses.** The recombinant baculoviruses AcRhPV6, AcRhPV7, AcRhPV11 and AcRhPV12 were generated according to the manufacturer’s protocols. Transfection experiments were carried out in Sf9 cells using lipofectin
(Invitrogen). Seven days post transfection, cell supernatants were harvested and recombinant viruses were isolated by two rounds of plaque purification in Sf21 cells. Individual plaque isolates were later amplified following confirmation of RhPV expression by immunoblot analysis of the infected cell lysates using purified polyclonal RhPV antiserum. The titers of virus stocks were determined by plaque assay using standard procedures (O’Reilly et al., 1992). AcRhpV-6 was used in further experiments.

**Infection of Sf21 cells.** Sf21 cells (3x10⁶ cells in a 25 cm² flask) were infected with recombinant AcRhpV-6 or the control virus AcRP231lacZ at multiplicity of infection (m.o.i) of 5 or 10. After 1 h at room temperature, the virus was removed and 5 ml of cell growth medium was added to the flasks and incubated at 28°C until cytopathic effects (CPE) were observed in the cells, typically 3-4 days post infection (dpi), at which time the cells were harvested for further analysis.

**Purification of virus-like particles.** Virus particles were purified from Sf21 cells infected with recombinant AcRhpV-6 or AcRP231lacZ, at 5 dpi as described by Krishna et al. (2003). Purified virus particles were negatively stained with 2% uranyl acetate and examined by TEM.

**Northern blotting.** Total cellular RNA was purified from Sf21 cells infected with AcRhpV-6, at 4 and 5 dpi and AcRP231lacZ infected cells at 5 dpi using Trizol (Invitrogen) according to the manufacturer’s specifications. RNA was extracted from purified VLPs using the Absolutely RNA® RT-PCR miniprep kit (Stratagene) according to the manufacturer’s directions. The RNA concentration was determined by measurement of absorbance at 260 nm. RNA was analyzed by Northern blot as described by Koev et al. (1999). Approximately 100 ng of RhPV RNA was run as a positive control. A ³²P-labeled
probe complementary to the 1.9-kb 3'-terminal sequence of RhPV was used to detect positive-sense RNA in the cells.

**Detection of RhPV RNA inside the VLPs generated by recombinant baculovirus.** To detect RhPV RNA packaged in the VLPs, RT-PCR was employed using the primers 5'-CAGAACTAGGCCTTCAGCTT-3' (6460 bp) and P6 (10011 bp), 5'-GGGGGGTACCGACGAAAAATGACATTACAG-3', resulting in a 3.5 kb RT-PCR product.

**Immunogold electron microscopy.** Sf21 cells (3x10^6 cells per 25 cm^2 flask) were infected with AcRhPV-6 or AcRP231lacZ at 10 p.f.u per cell, and the cells were harvested 5 dpi. Preparation of samples and treatment of grids was previously described (Chapter 2).

**Establishment of virus free aphid colonies.** Clones of uninfected *R. padi* were initiated parthenogenetically from single apterous adults. Progeny of each family were tested for the presence of RhPV by western blot and RT-PCR. The vertical transmission rate of RhPV in *R. padi* is approximately 28% (D'Arcy et al., 1981). Several virus-free aphid clones were obtained. These virus free colonies and RhPV infected aphid colony were maintained in separate buildings.

**Aphid infectivity assays.** Acquisition of the virus on aphid feeding was conducted as previously described (Rasochova, 1996, 1997). Briefly, for membrane feeding acquisition of virus or VLPs, aphids were allowed to feed for 16 h on purified virus preparations derived from one 25 cm^2 flask that had been infected with 10 p.f.u per cell and harvested 5 dpi with AcRhPV or AcRP231lacZ. 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 for a period of 6 weeks. Three replicates were carried out with at least 30 aphids per replicate and the infectivity of the VLPs was tested.

Aphids were tested weekly for RhPV infection from 2 weeks to 6 weeks after virus acquisition, using RT-PCR and western blot analyses. Total RNA from at least six aphids was extracted using the Absolutely RNA® RT-PCR miniprep kit (Stratagene). The procedure for RT-PCR is described above. The primers used were 5’-

CAGAACTAGGCCTTCAGCT-3’ (bp 6460) and P6 (10011 bp), 5’-

GGGGGTACCGACGAAAAATGACATTACAG-3’. PCR conditions used were 94°C for 1 min, 60°C, 1 min; 72°C for 4 min; 30 cycles and final extension at 72°C for 10 min. The aphids were also tested for virus infection by western blot analysis. At least six aphids from each treatment were ground in a microfuge tube in 200 µl of 1x protein dissociation buffer (2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM-Tris-HCl, 0.01% bromophenol blue, pH 6.8), boiled for 5 min and proteins resolved by SDS-PAGE. Approximately 150 ng of purified virus was run as a positive control. Ten microliter samples were resolved by electrophoresis on 4-12% SDS-PAGE gels (Bio-Rad Laboratories).

Results

Construction of recombinant baculovirus. A full length cDNA clone of RhPV was assembled by ligation of the three overlapping RT-PCR products (Fig. 2). Recombinant baculovirus transfer vectors AcRhPV1 through 12 were generated by using the Bac-to-Bac expression system. The resulting baculoviruses had a polyhedrin negative phenotype. When Sf21 cells were infected at 10 p.f.u per cell, CPE were observed at 4-5 dpi. The cells became sausage shaped and detached from the surface of the flask (Fig. 3B). Similar CPE were
observed in the cells infected with AcRP23lacZ, a polyhedrin negative virus (Fig. 3C). The mock-infected cells were healthy and there was an increase in the number of cells relative to the infected cells (Fig. 3A).

**Characterization of virus-like particles.** Following infection of Sf21 cells with AcRhPV-6, 26-27 nm diameter isometric particles (VLP) that were morphologically indistinguishable from RhPV particles were detected (Fig 4A). In addition to the 26-27 nm VLPs, 45-47 nm particles were also detected in cell lysates which were baculovirus nucleocapsids in cross section (Fig 4A and B). When the virus particles purified from Sf21 cells infected with AcRP23lacZ were observed by negative staining with 2% uranyl acetate, only the baculovirus nucleocapsids (45-47 nm) were observed (Fig 4B).

**Electron Microscopy Studies.** Ultrathin sections of Sf21 cells infected with AcRhPV-6 or AcRP23lacZ were analyzed using transmission electron microscopy. The nuclei of baculovirus infected cells contained many baculovirus particles (N, Fig 5A and 5B). Membranous nuclear vesicles were observed only in cells infected with AcRhPV-6. The nuclei of the baculovirus-infected cells contained electron dense particles, (V in fig 5A) that were present in the nucleus of AcRhPV-infected cells but not in AcRP23lacZ-infected cells. These particles may be RhPV VLPs. To confirm the identity of the putative VLPs, we conducted immunogold labeling of the sections of infected Sf21 cells. When purified anti-RhPV antibody was used for immunological analysis, intense labeling was observed in the nucleus of cells infected with AcRhPV-6, but not in the cytoplasm and the labeling was present specifically around the baculovirus nucleocapsids (Fig 6A). No specific labeling was observed in cells infected with AcRP23lacZ (Fig 6B).
Synthesis of RhPV RNA in recombinant baculovirus-infected Sf21 cells. To determine whether recombinant baculovirus expression would give rise to detectable levels of RhPV RNA in insect cells, Sf21 cells were infected with AcRhPV-6 or AcRP231lacZ at an m.o.i of 10. The cells infected with AcRhPV-6 were harvested at 4 and 5 dpi, while the cells infected with AcRP231lacZ were harvested at 5 dpi. Northern blot analysis was performed using a $^{32}$P labeled negative sense 3’ end RhPV RNA sequence (Chapter 2). RhPV RNA was not detected in RNA extracts of cells infected with AcRP231lacZ (Fig 7a, lane N). In contrast, RhPV RNA was detected in S121 cells infected with AcRhPV-6 at 4 and 5 dpi, (Fig 7a, lanes 3 and 4). The RhPV RNA ran higher in the gel than the 10 kb positive strand RNA (Fig 7a, lane P). Replication of RhPV RNA by the AcRhPV-6 infection on to Sf21 cells was unexpected because attempts to induce RhPV RNA replication in these cells by transfection with viral RNA were unsuccessful (Chapter 2). Hybridization with the negative-sense probe confirmed the presence of positive-sense RNA in the cells infected with AcRhPV-6. Several bands were detected in AcRhPV-6 samples. The major band was approximately 11.5 kb nucleotides, with a second band at approximately 10 kb. To determine whether RhPV RNA was packaged in the VLPs, RNA was also extracted from VLPs purified from infected Sf21 cells (harvested at 5 dpi). No RNA was detected by hybridization, and so samples were analyzed for the presence of RhPV RNA by RT-PCR. Figure 7b shows detection of RhPV RNA in VLPs by RT-PCR. The size of the RT-PCR product (3.5 kb) was similar that acquired from the positive control of viral RNA (Fig 7b, lane P), as expected based on the primers used for RT-PCR. These results indicated that genomic RhPV RNA was packaged into baculovirus-produced VLPs. However, only small quantities were packaged because we were unable to detect the RNA by northern blot hybridization. Moreover, our studies did
not show whether a complete RhPV genome, a larger RNA or a smaller transcript had been packaged. It is also unknown what proportion of baculovirus produced VLPs contained RhPV RNA.

**Infectivity of baculovirus-produced RhPV VLPs**

The goal of this set of experiments was to determine if the VLPs generated in AcRhPV-6 infected Sf21 cells were infectious to aphids. Virus-free aphid colonies were established from a single apterous female and the colonies were repeatedly tested for the virus by western blot analysis (Fig 8) and RT-PCR. Approximately 30 virus-free aphids were fed with the baculovirus-produced, purified VLPs (see Methods) for 16 h, then transferred on to healthy plants and reared for at least 6 weeks. The experiment was replicated three times. Aphids were found to be positive by RT-PCR and western blot analysis 4 weeks post feeding in one of two replicates. (Results from the third replicate are pending).

**Discussion**

We have shown that virus-like particles of RhPV can be expressed from a recombinant baculovirus. Electron microscope studies revealed no obvious morphological differences between the authentic and synthetic virus particles except to the intracellular location. Western blot analysis showed that the polyprotein is cleaved appropriately and immunoelectron microscopy showed that virus particles were assembled in the nucleus of the baculovirus infected cell. Royall et al. (2004) showed that the RhPV 5’ IRES functions efficiently in Sf21 cells and in the *in vitro* Sf21 cell-based lysate system (Kubick et al., 2003). The IGR IRES elements are unusual in that they direct translation initiation from non-AUG codons and they do not require any canonical initiation factors for assembly of
initiation complexes on the mRNA (Wilson et al., 2000; Nishiyama et al., 2003). So it is likely that both 5' and 3' polyproteins are synthesized and processed in Sf21 cells and subsequently assembled into virus-like particles in the nucleus.

Northern blot hybridization of the total cellular RNA extracted from the AcRhPV-6 infected Sf21 cells, revealed RNA species of a size larger than the RhPV genome (11 kb compared to the 10 kb genomic RNA), in addition to smaller transcripts (Fig 7a). The first base of the RhPV cDNA was positioned 105 bases downstream of the polyhedrin transcriptional start site. RNA transcription proceeds about 300-400 bases beyond the end of the RhPV sequence because of the lack of termination or polyadenylation signals in the viral genome. Addition of a poly (A) tail of 100 to 150 bases results in a transcript of larger size than the expected genome size (Schneemann et al., 1993). Elevated levels of such extended transcripts have been previously documented under conditions of impaired polyadenylation caused by mutations downstream of the AAUAAA hexanucleotide of the SV40 late polyadenylation signals (Breathnach, 1984; Kessler et al., 1987; Zhang et al., 1986; Mason et al., 1986).

The apparent low infectivity of RhPV VLPs to the aphids suggests that only a small proportion of the VLPs contain the complete genome. Alternatively, the presence of additional sequences at the 5' and 3' ends of the genome results in loss of infectivity. The infectivity studies should be repeated using large quantities of recombinant VLPs for feeding purposes. The synthesis of RNA from baculovirus vectors in the absence of replication may result in poor packaging efficiency. RNA replication may be required for efficient packaging, as for other viruses. The results suggest that there may be an encapsidation advantage for RNAs produced by replication rather than transcription. RNA synthesized by
replication in the cytoplasm is likely to be present in a more suitable conformation for packaging than RNA transcripts that are synthesized in the nucleus from the baculovirus genome (Krishna et al., 2003).

In summary, our results show that recombinant baculoviruses can be used to generate immunogenic virus-like particles of RhPV that show some degree of infectivity to aphids. The baculovirus expression system represents an alternative tool for use in the study of the assembly and structure of viruses. This is the first report of the use of the baculovirus expression system for production of a Dicistrovirus, and the first report of baculovirus production of an infectious heterologous virus.

Acknowledgements

This study was supported by an Iowa State University Carver Trust grant as well as Hatch Act and State of Iowa funds.

References


Fig 1. Genome organization of RhPV. The 10,011 nt RNA genome of RhPV encodes two polyproteins (gray boxes) with significant similarity to polyproteins of como- and picornaviruses. ORF 1 encodes the nonstructural proteins: RNA helicase (hel), cysteine protease (prot), and RNA-dependent RNA polymerase (pol). ORF 2 encodes the four structural proteins, VP1 through VP4. Molecular weights of the three proteins (VP1, VP2, VP3) that comprise each virion face (capsomere) are shown (K). The distinct IRESes located in the 5' UTR and intergenic region (IGR), are indicated.
Fig 2. Cloning strategy used to generate full-length cDNAs placed under the control of the polyhedrin promoter of the baculovirus expression vector pFastBac1. The positions of the PCR primers in relation to the RhPV RNA sequence are indicated by the arrows. Dotted arrows at 5' and 3' ends indicate the introduced restriction sites (EcoRI and KpnI respectively). Three cDNA fragments (indicated by open, dark and grey rectangles) were produced and introduced into pFastBac1 in the order shown in the figure. Numbering refers to the nucleotide positions in RhPV RNA.
Fig 3. CPE following infection of Sf21 cells with AcRhPV-6. A, Mock infected Sf21 cells, cells are closely packed and healthy indicated by arrow. B, CPE in the cells infected with AcRhPV-6 (5 dpi) at 10 p.f.u. per cell. Infected cells are sausage shaped (indicated by arrows), cell number is reduced, and 90% of the cells float on the surface. C, Similar CPE are observed when the cells are infected with polyhedrin negative virus, AcRP23LacZ.
Fig 4. Examination of baculovirus expressed VLPs. A, electron micrograph of negatively stained RhPV VLPs recovered from *S. frugiperda* cells (Sf21) infected with AcRhPV-6 recombinant baculovirus. There are particles of two different sizes, baculovirus nucleocapsids (N) and RhPV VLPs (V). B, electron microscopy of purified particles from Sf21 cells infected with AcRP23lacZ, with only nucleocapsids (N) detected. Bar represents 200 nm.
**Fig 5.** Electron micrographs of thin sections of Sf21 cells infected with recombinant baculoviruses and harvested 5 dpi. Panel A cells infected with AcRhPV-6. Bar represents 200 nm. Panel B cells infected with AcRP231lacZ. Bar represents 500 nm. Baculovirus nucleocapsids (N), membranous vesicles in the nucleus (M), and RhPV VLPs (V) are indicated by arrows.
Fig 6. Examination of sections of Sf21 cells infected with AcRP23LacZ or AcRhPV-6. (A) Sf21 cell infected with AcRP23LacZ. (B), Immunogold labeling (L) of Sf21 cell infected with AcRhPV6. RhPV specific antibody binding in the nucleus was detected by goat anti-rabbit serum conjugated with 10 nm colloidal gold. Baculovirus nucleocapsids (N) and clumps of gold label in B (L) are indicated by arrows. Bars represent 200 nm.
Fig 7. Detection of RhPV RNA in recombinant baculovirus infected cells and baculovirus-produced VLPs. A, Northern blot hybridization of total cellular RNA of Sf21 cells infected with 10 p.f.u. per cell of AcRhPV-6, 4 dpi (lane 3) and 5 dpi (lane 4); AcRP23lacZ (N); 100 ng of viral RNA (P). A $^{32}$P labeled negative sense 3' end RhPV sequence was used as a probe. Arrows show transcript of approximately 11 kb, and 10 kb viral RNA control (P). Bottom panel represents RNA loading control.

B, RT-PCR results for the RNA extracted from purified VLPs. M, 1 kb DNA ladder; P, RhPV RNA; N, RNA extracted from purified baculoviruses from AcRP23lacZ infected Sf21 cells; V, RNA extracted from purified baculovirus and RhPV VLPs from AcRhPV-6 infected Sf21 cells.
Fig 8. Detection of RhPV coat proteins by western blot in infected and virus free aphid colonies. RhPV virions (W, 0.1 μg); RhPV-positive *R. padi* colony (+); virus-free *R. padi* colony (-); *Aphis pisum* negative control (C). n = 6 aphids for each lane.
CHAPTER 4

INFECTIOUS IN VITRO TRANSCRIPTS OF RHOPALOSIPHUM PADI VIRUS SYNTHESIZED FROM A FULL-LENGTH cDNA CLONE

Sandhya Boyapalle,¹ Randy C. Beckett,² W. Allen Miller,² Bryony C. Bonning,¹

¹Departments of Entomology and ²Plant Pathology, Iowa State University, Ames, IA-50011

Paper to be submitted to Journal of Virology

ABSTRACT

Here, we report construction of a full-length infectious clone of *Rhopalosiphum padi* virus (*RhPV*; *Dicistroviridae*). A full-length cDNA was constructed from reverse transcription PCR products derived from virions isolated from the bird cherry-oat aphid *Rhopalosiphum padi*. The full-length cDNA was cloned into the plasmid pGEM3ZF under the control of a T7 promoter and amplified in *Escherichia coli* DH5a. RNA transcribed from the full-length cDNA clone was infectious upon transfection into the insect cell lines Z10-2 (glassy winged sharp shooter, *Homalodisca coagulata*) and DMII (corn leaf hopper, *Dalbulus maidis*) cells, resulting in production of progeny virus that was phenotypically indistinguishable from the parent virus. The *in vitro* transcript caused the same cytopathic effects (CPE) as those caused by transfection of cells with the viral RNA. The virus-like particles purified from cells transfected with the transcript of the full-length clone were infectious to Z10-2 cells and also to *R. padi*. The infectious cDNA clone of RhPV, together with the cell culture system, will provide valuable experimental tools for study of the replication and pathogenesis of this dicistrovirus.
INTRODUCTION

Rhopalosiphum padi virus (RhPV) belongs to the newly-defined *Dicistroviridae* family (ICTV, 2004). This emerging virus group includes the *Drosophila* C virus (DCV), *Plautia stali* intestine virus (PSIV) (Sasaki et al., 1998), and cricket paralysis virus (CrPV). These viruses are however, related to picornaviruses in protein sequence and structure, but differ in genome organization, and translation control signals (Tate et al., 1999) (Fig. 1). Like picornaviruses, dicistroviral genes are expressed from large polyproteins that are cleaved into functional proteins at specific sites by host and viral proteases (Liljas et al., 2002). The RhPV genome is composed of a positive polarity ssRNA of about 10011 nucleotides (nt), containing two open reading frames (ORFs) with the structural proteins encoded in a separate, 3'-proximal ORF (Fig. 1). Proteins from both ORFs are translated via internal initiation of ribosomes directly on the genomic RNA at internal ribosome entry sites (IRES) (Wilson et al., 2000b). Viruses with similar genome organization have not been isolated from vertebrates.

Dicistrovirus RNA has a genome-linked protein at the 5' end instead of a normal cap structure, and the internal ribosome entry sites (IRESes) allow for very efficient cap-independent translation of each ORF. IRESes from picornaviruses and other RNAs have been well-studied (Hellen & Sarnow, 2001). The 5' IRES of distroviruses needed for translation of ORF 1 and the intergenic region (IGR) IRES that facilitates translation of ORF 2 are unrelated to each other and to any other known IRESes. The 5' IRES is at most 200 nt long, functions in cells from all kingdoms, but is still structurally ill-defined (Woolaway et al., 2001). In contrast, the IGR IRES was rapidly characterized as it provides a remarkable
new way for ribosomes to enter an mRNA (Domier et al., 2000; Wilson et al., 2000b; Domier & McCoppin, 2003). The IGR IRES forms a complex pseudoknotted structure that mimics a tRNA base paired to the mRNA in the ribosomal P site. This causes the entering ribosome to immediately begin the elongation process of translation on ORF 2, and omit the highly regulated, complex process of initiation of translation (Wilson et al., 2000a). Thus, there is no AUG- or any other start codon. This process allows the virus to bypass host antiviral translation regulatory mechanisms. Like the IRES of cricket paralysis virus (CrPV), the Taura syndrome virus (TSV) IRES can assemble 80S ribosomes in the absence of initiation factors and initiation takes place at non-AUG codons (Cevallos and Sarnow, 2005).

Infectious cDNA clones of RNA virus genomes are valuable tools used to gain insight into genetic expression and the study of viral life cycles. The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into the viral genome (Pekosz et al., 1999). The production of cDNA clones from which infectious RNA can be transcribed, is an important step in the development of reverse genetics systems for all positive-strand RNA viruses. This approach has also increased our understanding of molecular mechanisms of plant-virus interactions, cell-to-cell movement of viruses, and has resulted in the development of anti-viral vaccines (Boyer & Haenni, 1994).

The nucleotide sequences of many dicistroviruses have been determined and production of infectious transcripts of black queen cell virus (BQCV) has been reported (Benjeddou et al., 2002). Here we describe the construction of a full-length clone of RhPV, and production of in vitro transcripts that are infectious to cells derived from the glassy-
winged sharp shooter *Homalodisca coagulata* (Z10-2) (Kamita et al., in press) and corn leafhopper, *Dalbulus maidis* (DMII) (McIntosh, unpublished data). The virus-like particles derived from RNA transfections into the insect cells, were also infectious to aphids.

**MATERIALS AND METHODS**

**Cells and viruses.** RhPV was purified from infected colonies of *R. padi* maintained as previously described by D’Arcy et al. (1981). Cell lines derived from corn leaf hopper, *Dalbulus maidis* (DMII) (A. McIntosh, University of Missouri, Columbia), and glassy winged sharp shooter, *Homalodisca coagulata* (Z10-2) (Kamita et al., in press) were maintained in Excel 405 (JRH Biosciences), supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma).

**Construction of a full-length cDNA clone for RhPV.** Construction of the full-length cDNA clone of RhPV in the baculovirus expression vector (pAcRhPV-6) is described in Chapter 3. In this study, a full-length RhPV clone was generated from the pGEM3ZF vector, which has T7 and SP6 promoter sequences flanking the multiple cloning site. The full-length cDNA was cut from pAcRhPV-6 using EcoRI and KpnI and ligated in to pGEM3ZF. The full length clone is 10,021 nt in length excluding the poly(A) tail and contains ten non-viral bases at the 5’ end.

**In vitro transcription of full-length RhPV RNA.** *In vitro* transcription of the above described cDNA clone (pRhPV-6) was carried out using T7 RNA polymerase (MEGAscript™ high yield transcription kit, Ambion). Briefly, the plasmid DNA (pRhPV-6) was extracted using a plasmid miniprep kit (Bio-Rad) according to the manufacturer’s specifications. The plasmid containing full length RhPV cDNA was digested with Acc651 to produce a linear DNA template. RNA was transcribed by the manufacturer’s specifications.
using 1 μg of linear DNA template to synthesize the full-length positive-sense RNA transcript.

Full length negative-sense RNA was acquired by linearizing the plasmid with EcoRI, and used as template for in vitro transcription from the SP6 promoter using SP6 Megascript kit (Ambion). The transcription mixture was incubated at 37°C for 2 h and then digested with Dnase I (provided with the transcription kit) for 30 min at 37°C. The reaction mixture was extracted using phenol-chloroform and then subjected to a second round of Dnase I digestion, phenol-chloroform extraction to avoid carryover of template DNA with the transcripts. The transcripts were ethanol precipitated at -20°C for 2 h. The RNA pellet was washed with 70% ethanol, dried and resuspended in 100 μl of RNA suspension solution (Ambion). RNA was analyzed after each extraction by electrophoresis in 0.8% agarose gels, followed by ethidium bromide staining. RNA concentration was determined by A260 measurements.

**In vitro translation.** In vitro translation reactions with 35 S-met labeling (25 μl) using wheat germ extract were conducted according to the manufacturer’s protocols (Promega). Translation reactions with full-length RhPV transcripts using 10 μg RhPV-6 transcript or viral RNA contained 128 or 153 mM potassium acetate. Five microliters of the translation product was examined by SDS-PAGE (10% gel) (Wang & Miller, 1995). The gels were dried and imaged by using a Phosphoimager (Molecular Dynamics).

**RNA transfection.** Z10-2 and DMII cells were maintained in Excel 405 medium (JRH Biosciences) containing 10% FBS. Cells at ~70% confluency were used for RNA transfection. Ten micrograms of in vitro-produced positive-sense transcript, negative-sense
transcript or viral RNA were transfected using DOTAP according to the procedure previously described (Masoumi et al, 2003).

Cytopathic effects (CPE) were observed in Z10-2 cells 4 days post transfection (dpt) and in DMII cells (8-10 dpt). The cells were harvested at 0, 12, 24, 48, 72, 96, 120 hpt for extraction of total RNA.

**Northern blot hybridization.** To analyze the production of RNA over the course of infection, Z10-2 cells were transfected with RhPV-6 full length positive-sense RNA. Transfection of cells with full length negative-sense RhPV transcript (non replicatable RNA) was used as a negative control in this experiment. Transfected cells were harvested at time points 0, 0.2, 12, 24, 48, 72, 96, 120 hpt and total cellular RNAs were extracted using Trizol (Invitrogen) according to the manufacturer’s specifications. RNA was analyzed by Northern blot as described by Koev et al. (1999). Approximately 100 ng of the viral RNA or full-length negative sense RhPV transcript were run along side as positive controls. A $^{32}$P-labeled probe complementary to the 1.9-kb 3’-terminal sequence of RhPV (plasmid digested with HpaI and transcribed using SP6 RNA polymerase) was used to detect production of positive strand RNA in the cells. A $^{32}$P-labeled probe complementary to the 1.2-kb 5’ end sequence of RhPV (plasmid digested with HpaI and transcribed using T7 RNA polymerase) was used to detect negative-sense RNA inoculum in the cells.

**Detection of antisense RhPV RNA.** The cells transfected with RhPV-6 positive sense transcript were analyzed at 0, 24, 48, 72, 96 hpt for the presence of the antisense RNA replicative intermediate using strand-specific RT-PCR using the primers at positions 7476 and 8780 of the RhPV sequence (Moon et al, 1998). Negative sense, full length in vitro transcript served as positive control, where as viral RNA served as a negative control.
Reverse transcription (RT) with the sense primer was carried out using Superscript RT (Gibco-BRL) according to the manufacturer's specifications. Following cDNA synthesis, the RNA was degraded by digestion with RNase A (1 μg, Promega). The reaction mixture was then extracted once with phenol-chloroform and precipitated with ethanol. The cDNA was then PCR amplified with sense and antisense primers using ExTaq polymerase (Invitrogen). “No RT controls”, i.e. controls for PCR reactions without the RT reaction were also run along side to show that PCR products resulted from RNA and not from the pRhPV-6 DNA plasmid, which was a potential contaminant from transfections with the RhPV-6 positive sense transcript.

**Purification of virus-like particles (VLP).** Z10-2 cells transfected with RhPV-6 transcript were harvested when extensive CPE was observed (typically at 4 dpt) and the virus particles were purified as described by Krishna et al (2003). Purified virus particles were negatively stained with 2% uranyl acetate and examined by TEM.

**Immunocytochemistry of Z10-2 and DMII cells transfected with RhPV-6 transcript.** Z10-2 cells and DMII cells were grown to ~70% confluency in T-25 flasks (Fisher Scientific). The cells were transfected with 1μg of DOTAP and 20 μg of RhPV-6 RNA. The cells were harvested 4 dpt (Z10-2) cells and 10 dpt (DMII cells). The cells were harvested, fixed and treated as described in Chapter 2.

**Experimental infection of *R. padi.** Clones of uninfected *R. padi* were generated as described in Chapter 3. Acquisition of the virus on aphid feeding was conducted as previously described (Rasochova, 1996, 1997). Briefly, for membrane feeding acquisition of VLPs, aphids were allowed to feed for 16 h on purified virus preparations derived from one 25 cm² flask infected with 20 μg of RhPV-6 positive sense transcript (as described in chapter
Three replicates were carried out with at least 30 aphids per replicate. The infectivity of the VLPs was tested as follows: Aphids were tested weekly for RhPV infection from 2 weeks after virus acquisition, using RT-PCR and western blot analyses as described in chapters 2 and 3.

**Analysis of RhPV infection of aphids by light microscopy.** For immunolocalization studies by light microscopy, aphids fed with VLPs generated from RhPV-6 transfection in Z10-2 cells were fixed (6 weeks post feeding). Aphids were bisected into head and abdomen with a razor blade while submerged in fixative (1% paraformaldehyde, 0.5% gluteraldehyde in 0.05 M sodium cacodylate buffer pH7.1). The abdomens were fixed in 2% paraformaldehyde-2.5% gluteraldehyde-0.05% sodium cacodylate, pH7.1, for 30 min at 4°C. After washing the cells three times (10 min each time) with 0.05M sodium cacodylate, the bodies were dehydrated with a series of ethanol concentrations (50%, 70%, 85%, 95%, 3x100%) for 30 min at each step at 4°C. Abdomens were then infiltrated with ethanol: LR White Resin (London Resin Company Ltd, England) using ratios of 1:1 (2 h at 4°C), and 1:3 (overnight at 4°C) followed by pure LR White (24 h at 4°C) changing resin every 12 h. The abdomens were later embedded in gelatin capsules, and resin was polymerized at 4°C for 48 h with the UV light on (Vandenbosch, 1991).

LR-White embedded sections were placed on Probe-On Plus slides (Fisher Scientific) with a drop of distilled water and dried for an hour at 50°C on a warming tray. Sections were treated with 25 μl of TBS-Tween supplemented buffer (0.05M Tris, 0.85% NaCl, pH 8.3-8.5, 0.5% normal goat serum, 0.5% normal pig serum and 0.5% BSA, 0.05% Tween-20) with 3% non-fat dry milk for 2 h at room temperature. Sections were then treated with 50-100 μl drops of 1:10 purified rabbit polyclonal anti-RhPV antiserum (described in Chapter 2) diluted
in TBS-Tween supplemented buffer with 3% non-fat dry milk for 4 h at 37°C. The slides were stream washed with TBS-Tween buffer, and then washed 3x15 min in TBS-Tween supplemented buffer. The sections were then treated with 25 μl drops of 1:100 dilution of secondary goat anti-rabbit antibody conjugated with 10 nm gold particles (Ted Pella Inc.) for 1 h at room temperature. After stream washing and three washes in drops of distilled water (15 min each wash), silver enhancement of the gold-immunolabeled slides was carried out using silver enhancement goldmark kit (Electron Microscopy Sciences) according to the manufacturer’s specifications. The slides were finally dried, immersed briefly in Xylene, and coverslipped with Permount. The slide without immunolabeled sections was used as a control for non-specific silver precipitations.

RESULTS

Production of RhPV transcripts. An RhPV cDNA clone (pRhPV-6) encompassing nucleotides 1 to 10011 with 10-bp of non viral residues (GAATTC AT A AG) at the 5’ end was transferred from pAcRhPV-6 (described in Chapter 3) into pGEM3ZF (Invitrogen). The RhPV sequence is flanked by a T7 promoter sequence at the 5’ end and an SP6 promoter sequence at the 3’ end. In vitro transcription of Acc651-linearized pRhPV-6 driven by T7 polymerase generated a transcript of ~10 kb as determined by denaturing agarose gel electrophoresis and northern blot analysis (Fig. 4 A). The transcript is expected to have 13 nt of non-viral bases at the 5’ end and 1 non-viral base at the 3’ end. We call the in vitro transcript from this plasmid, RhPV-6. The transcription of EcoRI cut pRhPV-6 driven by SP6 polymerase always generated two transcripts of ~9.8 to 10 kb (Fig. 4B).

Translation in vitro. To test if viral proteins would be synthesized as expected, we translated the full length transcript in vitro. Using wheat germ extract, translation of the
RhPV-6 transcript was compared with that of viral RNA (Fig. 2). In both cases, the $^{35}$S-met labeled protein migration patterns were identical. Under the conditions used, a band of ~90 kDa was always observed, which corresponds to the expected mass of the uncleaved polyprotein translated from ORF 2 (Fig. 1). Thus wheat germ extract appears to lack activity necessary to process viral coat proteins. Translation of ORF 1 was not detected. This large polyprotein (229 kDa) would not have been detected by using this system.

**Detection of CPE in the transfected cells.** Z10-2 cells transfected with RhPV-6 transcript showed CPE by 4 dpt. The cells lost their characteristic fibroblast-like extensions, became rounded and were of uniform size. Approximately 90% of the cells detached from the surface (Fig. 3). Similar CPE was observed when the cells were transfected with viral RNA (Fig. 2, Chapter 2). There was evidence of cell lysis and on examination with a phase contrast microscope the cells had a granular appearance. In contrast, cells in the control wells were healthy with characteristic fibroblast-like extensions. For the DMII cell line, the cells exhibited CPE at 8-10 dpt. The cells were rounded and looked strikingly different from cells in the control wells. Infected cells were clumped and lost their surface extensions (data not shown). The control wells were crowded with cells.

**Synthesis of positive sense RNA following transfection of Z10-2 cells with RhPV-6 transcripts.** Northern blot hybridization was used to examine accumulation of positive sense genomic RNA molecules (Fig. 4A). Input positive-sense viral RNA and negative-sense *in vitro* transcript were detected at 12 and 24 hpt (Fig 4A and 4B). RhPV RNA could be detected at 2, 3, 4 dpt when cells were transfected with positive sense RhPV-6 transcript (Fig. 4A). In contrast, in cells transfected with non-replicatable RNA (negative-sense
inoculum) RNA levels decreased rapidly after 24 hr (Fig. 4B). Negative-sense transcript always migrated as a doublet.

**Detection of replicative negative-sense RNA.** Replication requires synthesis of negative sense viral RNA in the cell. Negative strands of the RhPV genome were detected by strand-specific RT-PCR in RhPV-6 transfected Z10-2 cells (Fig. 5). The cells were positive for the presence of antisense strands at 48, 72, 96 hpt. No PCR product was detected at 0 and 12 hpt indicating that RNA was synthesized in the cells at ~48 hpt. Positive sense RNA was more abundant than the negative-sense strand.

**Detection of virus-like particles (VLPs) in infected cells.** To determine whether virions could be observed in the infected cells, Z10-2 cells were transfected as described above with the RhPV-6 positive sense transcript, and the cells harvested at 4 dpt for purification of virus particles when CPE were pronounced (Krishna et al, 2003). Purified virus particles were negatively stained with 2% uranyl acetate and examined by TEM (Fig 6B). RhPV particles purified from aphids (Fig 6A) were examined for comparison. The size of the particles was estimated to be 26 - 27 nm. The synthesized VLPs and the native RhPV virions were indistinguishable.

**Ultrastructural analysis of cells transfected with the RhPV-6 transcript.** Z10-2 and DMII cells were transfected with the RhPV-6 transcript, harvested 4 and 10 dpt respectively and sectioned for examination by TEM. Immunogold labeling experiments showed that the RhPV antibody specifically labeled the amorphous ovoid inclusions (Fig. 7 c, d, g, h), thus indicating that viral coat proteins are associated with these structures in the cytoplasm. Occasionally, a clump of label was observed in infected cells associated with the cell membrane (Fig. 7f), which may indicate a site of viral endocytosis or release. Mock
infected Z10-2 or DMII cells did not exhibit any cytoplasmic inclusions or labeling (Fig. 7 a, b).

**Infected VLPs generated from transfected Z10-2 cells.** We conducted experiments to determine if the VLPs generated in RhPV-6 transfected Z10-2 cells were infectious to aphids. Virus-free aphid colonies were established from a single apterous female and the colonies were shown to be virus-free after repeated tests for the virus by western blot analysis and RT-PCR (Chapter 3). Inoculated aphids were found to be virus-positive by western blot analysis (Fig 8), two weeks post feeding, in all three trials. Aphids infected by VLPs were fixed and sectioned and purified anti-RhPV antibodies used for detection of virus using bright field light microscopy. Silver-enhanced immunogold labeling was observed in the midgut of the aphid 6 weeks post acquisition feeding (Fig 9 C). A small amount of labeling was also observed in the hindgut of the virus infected lab colony (Fig 9 A). No labeling was detected in virus free aphids (Fig 9 B).

**DISCUSSION**

We report the construction of a full-length cDNA clone of the RhPV genome. Several types of observations indicate that transcript RhPV-6 was infectious to both Z10-2 and DMII cells. CPE observed in the cells transfected with the RhPV-6 transcript was similar to that seen with the viral RNA transfections (discussed in Chapter 2). Although there was no striking increase in the amount of viral RNA detected in Z10-2 cells (Fig. 4A), in comparison with cells transfected with the non replicatable negative-sense RNA (Fig. 4B), detection of the negative-strand RNA by RT-PCR demonstrated that viral replication occurred in Z10-2 cells transfected with the RhPV-6 transcript. Negative strand RNA was detected by strand-specific RT-PCR at 48, 72, and 96 h (Fig. 5). By contrast, negative strand
RNA was detected from Z10-2 cells transfected with uncloned viral RNA from 12 hpt by RT-PCR. (Fig. 4, Chapter 2). The lower infectivity of the RNA transcript, relative to viral RNA is typical for transcripts of a number of other viruses (Lai et al., 1991; Sit & AbouHaidar, 1993; Khromykh & Westaway, 1994; Gristum & Gould, 1995; Iwamoto et al., 2001). Benjeddou et al. (2002) showed that the infectivity of RNA transcripts generated for BQCV was 350-fold lower than the infection efficiency of viral RNA, although the recovered virus was as infectious as the wild type.

Low infectivity of the transcript may be explained by the presence of 13 non-viral bases at the 5' end and the absence of the poly(A) tail that is present on the natural viral genome. The transcript also lacks a 5' cap (m7GpppG) structure which is required for optimum infectivity, probably because it enhances translation initiation or it improves mRNA stability by conferring greater resistance to host cell nucleases (Boyer & Haenni, 1994). All three of these differences from wild type virus are likely deleterious to specific infectivity of the transcript. Even short 5' non-viral extensions usually greatly inhibit replication of transcripts (Bujarski & Miller, 1992). Poly(A) tails are essential for efficient translation (Gallie, 1991; Tarun & Sachs, 1995; Preiss & Hentze, 1998) so the absence of a poly(A) tail is likely to hinder virus gene expression. The RhPV-6 transcript may be infectious without the poly(A) tail due to A-rich tract (16 nt) near the 3' end. However, based on its presence on the natural viral RNA (Moon et al., 1998), it is likely that addition of a 60-70 nt poly(A) tail will increase infectivity. The infectivity of RhPV-6 may be improved by removing the extra 5' bases.

Our EM observations (Fig. 7) revealed formation of electron dense amorphous cytoplasmic structures that were strongly labeled by anti-RhPV antibodies. Similar
structures were observed within the cytoplasm of these cells when transfected with viral RNA (Fig. 6, Chapter 2). In host specificity studies of RhPV in aphids conducted by Gildow and D’Arcy (1990), RhPV particles occurred free in the cytoplasm and also packed in crystalline arrays in large membrane vesicles.

The virus recovered from cells transfected with the in vitro transcribed RNA molecules was found to be infective to Z10-2 cells and to aphids. Bright field light microscopy studies of aphid bodies fed with the virus preparations revealed that the virus is infectious, but the degree of infectivity cannot be compared to that of wild type RhPV. However, enhanced labeling was observed in the midgut of both the virus positive colony and the aphids infected with the RhPV6 transcript-derived virions. Gildow and D’Arcy (1990) observed virus in the midgut of all the aphids tested (8/8) and frequently in the hind gut (4/8).

The development of an infectious clone will open new opportunities to conduct studies directed at understanding the molecular biology, persistence, pathogenesis and interaction of the virus with the host insect.

This study was supported by an Iowa State University Carver Trust grant as well as Hatch Act and State of Iowa funds.
REFERENCES


FIG 1. Construction of the full-length cDNA clone of RhPV. Genome organization of RhPV and its encoded proteins are shown at the top. Overlapping cDNA segments of RhPV were generated by RT-PCR and cloned into pFastBac1 (Fig 2, chapter 3) to generate AcRhPV6. This plasmid was digested with EcoRI and KpnI, and the resulting segment (10 kb) was cloned into pGEM3ZFvector to obtain plasmid pRhPV-6. The plasmid was used to generate positive sense and negative sense RNA transcripts with T7 promoter at 5’ end and SP6 promoter at the 3’ end of the genome.
FIG. 2. *In vitro* translation of the full-length transcripts and the viral RNA in wheat germ extract. Lane M is the BMV RNA provided as positive control, (1) no RNA control, (2 and 3) translation products of 10 µg of RhPV-6 in vitro transcript at 128 and 153 mM concentrations of potassium acetate respectively, (4 and 5) translation products of 10 µg of viral RNA at 128 and 153 mM potassium acetate concentration.
FIG 3. Z10-2 cells transfected with RhPV-6 transcript. (A) Z10-2 cells treated with DOTAP alone, negative control; (B) Z10-2 cells transfected with 10μg of RhPV-6 transcript and the CPE observed 4 days post transfection (dpt). Infected cells rounded (shown by arrows) and were of uniform size. 90% of the cells floated from the surface and the cells lost the fibroblast like extensions. The uninfected cells (A) possessed abundant fibroblast like extensions and the number of cells increased.
FIG 4. Detection of RNA following transfection of Z10-2 cells with the RhPV-6 transcript. (A) Z10-2 monolayer was transfected with 10 μg of RhPV-6 RNA using DOTAP, incubated at 28°C until harvesting at 0, 12, 24 h, 2, 3, 4, 5 dpt. Following extraction, total cellular RNAs were resolved on a formaldehyde gel along with the 100 ng RhPV-6 RNA (+), mock infected Z10-2 cells (-) and transferred to nylon membrane. Positive-sense RNA was detected by Northern blot hybridization using an in vitro synthesized 32P-labeled 3' end negative sense RNA probe. The positive sense RNA detected at 12 and 24 hpt was derived from the inoculum. (B) Z10-2 monolayer was transfected with 10 μg of negative sense full length RhPV transcript synthesized in vitro, incubated at 28°C until harvesting at 12, 24 h, and 2, 3, 4 dpt. Following extraction total cellular RNAs were resolved on a formaldehyde gel along with the 100 ng full-length negative-sense RhPV transcript synthesized in vitro (+), 100 ng full length positive sense RhPV transcript synthesized in vitro as negative control (-). RNA was detected by Northern blot hybridization using in vitro synthesized 5' end positive sense RNA probe. The negative sense RNA at 2, 3 dpt appeared to be inoculum degrading with the time. Bottom panels represent RNA loading control.
FIG 5. Detection of the negative-sense RhPV-6 transcript in infected cells. Strand-specific PCR was carried out on total RNA extracted from Z10-2 cells transfected with the positive-sense RhPV-6 transcript. Cells were harvested at the hpt indicated. Antisense RNA was detected at 48, 72 and 96 hpt. Also shown, 1 kb DNA ladder (L); mock infected Z10-2 cells (M); negative control using positive strand virion RNA as template, (-); negative sense, full length in vitro transcript as positive control (+). "No RT controls": controls for PCR reactions without reverse transcriptase on RNA extracted from infected cells show that PCR products are from RNA and not potential contaminating pRhPV-6 DNA plasmid.
FIG 6. TEM of the VLPs purified from Z10-2 cells transfected with RhPV-6. (A) RhPV virus particles purified from *R. padi* by 10-40% sucrose gradients were negatively stained with 2% uranyl acetate and analyzed by transmission electron microscopy (TEM). (B) Virus particles purified from Z10-2 cells 4 dpt with the RhPV-6 transcript. The size of the particles in A and B was estimated to be between 26 and 27 nm. The size bar represents 200 nm.
FIG 7. Immunocytochemistry of ultrathin sections of Z10-2 cells. (a, b), mock infected Z10-2 and DMII cells respectively, did not exhibit any inclusion bodies in the cytoplasm; (c - e) Z10-2 cells 4 dpt with 10μg RhPV-6 transcript; (f - h), DMII cells, 8 dpt with 10μg RhPV-6 transcript; (c, d, g, h) have oval shaped electron dense inclusion bodies with the label (shown by arrows) in the cytoplasm; and (e, f) have intensive labeling with gold particles in the cytoplasm and label associated with the cell membrane. The size bar in a, c-f, h represents 200 nm; in b and g, 500 nm.
FIG 8. Detection of RhPV virion proteins in aphids by western blot. RhPV-positive aphids from lab colony (+; n = 8 aphids); aphids fed Z10-2 derived RhPV-6 virions (RhPV6; n = 8); aphids from RhPV-free colony (-; n = 15).
FIG 9. Bright field light microscopy showing immunolocalization of RhPV by silver enhancement in the midgut of the aphids. A, RhPV-positive aphid from the infected lab colony. B. Aphid from virus-free colony. C, Virus-free aphid fed with VLPs purified from Z10-2 cells transfected with RhPV-6 transcript. m, midgut, h, hindgut. Scale bars, 20 μm in A, and B; 100 μm in C.
CHAPTER 5

GENERAL CONCLUSIONS

Overview

*Rhopalosiphum padi virus* (RhPV) is an aphid virus placed under the Genus *Cripavirus* and Family *Dicistroviridae*. Previously it was considered a member Picornaviridae based on physicochemical properties. The 10,011-nt polyadenylated RNA genome was completely sequenced (Moon et al., 1998). Analysis of the sequence revealed the presence of two open reading frames (ORFs). Direct sequence analysis of the RhPV capsid proteins showed that ORF2, which represents the last 2900 nt, encodes the three structural proteins (28, 29, and 30 kDa). The predicted amino acid sequence of ORF2 is very similar to the corresponding regions of *Drosophila C virus*, *Plautia stali intestine virus*, and to a partial sequence from the 3' end of the cricket paralysis virus genome. ORF1 is preceded by 579 nt of noncoding RNA and the two ORFs are separated by more than 500 nt of noncoding RNA. Like picornaviruses, these regions function to facilitate the cap-independent initiation of translation of the two ORFs. When two IRESs are present in the same RNA, the ORF downstream of the second often is expressed at a much higher level than the first (Mountford and Smit, 1995).

RhPV infects several species of aphid, including the Russian wheat aphid and vectors of barley yellow dwarf virus (BYDV), but host range studies of RhPV have been limited. This dissertation discusses the identification of cell lines susceptible to RhPV by viral RNA transfections, baculovirus expression of RhPV and the production of an infectious *in vitro* transcript of RhPV.
Cell lines susceptible to RhPV. For rapid, synchronous infections, and to permit inoculation with RNA directly, we sought cell lines capable of supporting RhPV infection. Of the nine cell lines tested, only the homopteran cell lines supported replication of RhPV. CPE were observed following transfection on to the glassy-winged sharpshooter *Homalodisca coagulata* cell line Z10-2 at four days post transfection, and the corn leafhopper *Dalbulus maidis* cell line DMII at 8-10 days post transfection. Our results indicate that the factors required to support IRES-dependent initiation of translation, processing of viral products and packaging virus particles are available in Z10-2 and DMII cells.

Baculovirus expression of RhPV. RhPV RNA was extracted from purified virus and reverse transcription (RT)-PCR used to amplify three fragments of the genome. These fragments were sequentially assembled into pFastBac (baculovirus expression vector) to produce the full-length 10,011 nt RhPV genome. Nine clones were acquired. The structure of four of the clones was checked by restriction enzyme analysis and sequencing across the junctions (AcRhPV6, AcRhPV7, AcRhPV11, AcRhPV12). We have shown in Chapter 3 that virus-like particles of RhPV can be expressed from a recombinant baculovirus. Electron microscope studies revealed no obvious morphological differences between the authentic and synthetic virus particles. Western blot analysis showed that the polyprotein is cleaved appropriately and immunoelectron microscopy showed that virus particles are being assembled in the nucleus of baculovirus infected cells. Our results show that recombinant baculoviruses can be used to generate immunogenic virus-like particles of RhPV that contain RhPV RNA, and show some degree of infectivity to aphids. The baculovirus expression system represents an alternative tool for use in the study of the assembly and structure of viruses.
Construction of infectious RhPV clones. The plasmid pAcRhPV6 was cut with EcoRI and KpnI and the full length RhPV cDNA was cloned into pGEM with an upstream T7 promoter and was named pRhPV6. In vitro transcribed, full-length genomic RNA from plasmid pRhPV6 proved to be infectious. We call the RNA transcript from this plasmid, RhPV6. RhPV6 RNA transfected on to Z10-2 cells resulted in CPE that were apparent 4 dpt as for parent virus RNA transfections. Virus-like particles (VLPs) purified from the infected cells were visualized by negative staining and the presence of VLPs in the cells was confirmed by IEM with the anti-RhPV antiserum (Chapter 4). Infectivity was confirmed by detection of negative-sense RNA in transfected Z10-2 cells and by infection of aphids after feeding on infected cell extracts (below).

Infection of aphids with RhPV6 RNA. Aphids fed on VLPs from Z10-2 cells transfected with RhPV6 tested positive for infection by RT-PCR, immuno-localization by bright field light microscopy, and western blot, two weeks after acquisition in three out of three trials (Chapter 4). Thus, the VLPs are infectious virions. Although RhPV6 is infectious to aphids, the infectivity of the clone may be enhanced by minor genetic modification. The reduced infection efficiency could be explained by the presence of non-infectious DNA templates (amplicons) as a result of errors introduced in the RT and/or PCR reactions. The presence of extra nucleotides at the 5' and 3' ends of the in vitro-transcribed RNAs could have also negatively affected their infectivity (Lai et al., 1991; Sit & AbouHaidar, 1993; Khromykh & Westaway, 1994; Boyer & Haenni, 1994). The extraneous nucleotides could have been precisely trimmed or excluded by the expressed RNA-dependent RNA polymerase during replication (Khromykh & Westaway, 1994; Yao & Vakharia, 1998), such that the progeny virus was as infectious as the parent virus. Adding the
poly(A) tail at the 3' end, and capping of the RNA transcripts was not explored. It is widely accepted that a cap structure (m$^7$GpppG) is required at the 5' end of the transcripts for optimum infectivity, possibly because it enhances translation initiation or it improves their stability by conferring a greater resistance to host cell nucleases (Boyer & Haenni, 1994). In a few cases, however, both capped and uncapped transcripts have proved to be highly infectious (Boyer & Haenni, 1994). In contrast, uncapped transcripts were shown to be more infectious than capped transcripts for *Tomato bushy stunt virus* (Hearne *et al.*, 1990). It is not known if RhPV has a virus-encoded protein (VPg) or a cap structure at the 5' end of its genome. Therefore, it is not clear if adding the cap structure would have compensated for the lack of a VPg, as has been suggested for other viruses (Boyer & Haenni, 1994). The current transcript contains 13 non-viral bases at the 5' end and lacks the poly(A) tail that is present on the natural viral genome. Both of these differences from wild type virus are likely deleterious to viral replication.

Even short 5' non-viral extensions usually greatly inhibit replication of transcripts (Bujarski & Miller, 1992). Poly(A) tails are essential for efficient translation (Gallie, 1991; Tarun & Sachs, 1995; Preiss & Hentze, 1998) so the absence of a poly(A) tail is likely to hinder virus gene expression. The RhPV6 transcript may be infectious without the poly(A) tail due to an A-rich tract (16 nt) near the 3' end. However, based on its presence on the natural viral RNA (Moon *et al.*, 1998), it is highly likely that addition of a 60-70 nt poly(A) tail will increase infectivity.

**Future Research**

**Potential Use of RhPV for Aphid Management.** The long term goal for this project is to produce transgenic crop plants that are resistant to aphids by using RhPV as a delivery
system for insect toxins that are active in the aphid hemocoel. Because the aphid virus, RhPV infects and replicates in aphids, it may provide an appropriate delivery system for insect toxins that are active within the aphid hemocoel. Aphids that ingest virus particles produced by the transgenic plant would be infected by the engineered RhPV. The toxin produced upon replication of the virus in the aphid would be secreted into the hemocoel resulting in death of the aphid.

Having isolated an infectious clone of RhPV and identified two homopteran cell lines that support replication of RhPV, research will be conducted to determine the feasibility of producing transgenic plants that express RhPV, to deliver insect toxins into the hemolymph of aphids. Experiments are planned to optimize the infectivity of RhPV-6 by removing 13 non-viral bases at the 5’ end and adding a poly(A) tail at the 3’ end of the genome. The infectivity of viruses derived from infectious transcript (RhPV-6) will be compared to RhPV infectivity in aphids. An insect specific toxin gene will be introduced into the RhPV genome, and assembly and stability of the virus tested in planta. The development of aphid resistant transgenic plants would contribute to environmentally sustainable pest management and would confer significant economic benefit to U.S. agriculture.

**Fundamental Studies.** The availability of an infectious clone of RhPV makes it possible to study in detail the mechanisms of viral replication and pathogenesis. First the infectious clone of RhPV will be sequenced. RNA replication is error prone due to the minimal proof-reading activities of RNA dependent RNA polymerase with error rates 10 thousand times higher than those encountered during DNA replication. Error rates approximate the reciprocal of the number of nucleotides in the viral genome (Domingo and Holland, 1997;
Moya et al., 2000). This means that the genome of any individual RNA virus particle will contain an average of one mutation from consensus wild-type sequence for that virus species. Once the sequence of the infectious clone (RhPV6) has been determined, interaction of RhPV6 with host cells can be well studied by engineering specific mutations into the viral genome. Secondary structures may be predicted and the role of stem loop structures in replication and packaging of RNA can be determined by mutational studies. Moreover, a possible major factor in determining host specificity of the virus can be assessed by producing a series of mutants from the infectious clone. For example, influenza A viruses can be isolated from a variety of animals, but their host range is restricted. For example, human influenza viruses do not replicate in duck intestine, the major replication site of avian viruses in ducks. Amino acids at positions 226 and 228 of hemagglutinin (HA) of the H3 subtype are known to be important for this host range restriction. Vines et al. (1998) showed that the Ser-to-Gly mutation at position 228, in addition to the Leu-to-Gln mutation at position 226 of the HA of the H3 subtype, is critical for human virus HA to support virus replication in duck intestine.

Having acquired an infectious clone of RhPV, the relationship between RhPV and the bird cherry-oat aphid, *R. padi* and the molecular bases for host species specificity can be studied in detail. The approach used to engineer this clone is applicable to construction of full-length infectious cDNAs of other related viruses. The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as reverse genetics (Pekosz et al., 1999). Such reverse genetics systems have been developed for a number of positive-stranded
RNA viruses, including picornaviruses, caliciviruses, alphaviruses, flaviviruses and arteriviruses, whose RNA genomes range in size from ~7 to 15 kb (Yount et al., 2000). The production of cDNA clones and/or PCR-amplicons, from which infectious RNA can be transcribed in vitro, is an essential step in the development of reverse genetics systems for these viruses. The availability of these clones/PCR-amplicons has facilitated the study of the genetic expression and replication of RNA viruses by the use of mutagenesis, deletions and insertions and by complementation experiments. It has also enhanced the understanding of the molecular mechanisms of natural or induced RNA recombination and of host–virus interactions such as cell-to-cell movement.
REFERENCES


Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids. *Transgenic Research* 4, 18-25.


site which functions efficiently in mammalian, plant, and insect translation systems. *Journal of Virology* 75(21), 10244-10249.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my major professors, Drs. Bryony C. Bonning and W. Allen Miller for providing the guidance, education, support and above all for keeping my project on track. It has been an honor and pleasure working in both the Bonning and Miller labs. Throughout the last four years, I have always been learning and aspiring for new things and the journey towards my goal became easier with time. Thanks to all the members in the Bonning lab with whom I have enjoyed working: Sjiun Liu, Zhiyan Liu, David Sandgren, Tyasning Nusawardani, for helping me through difficult times. Special thanks to Sijun Liu for sharing his aphid expertise with me. Finally thanks to all the members in the Miller lab; William Staplin, Jackie Jackson, Aurelie Rakotondrafara, Ruizhong Shen, Elizabeth Pettit, for their thoughtful and helpful discussions when things became baffling. Special thanks to Randy Beckett for helping me learn many practical aspects when I first started my research work in the Miller lab.

Special thanks to Arthur Mcintosh, Shizuo George Kamita who provided the cell lines used in this work. Without their generous gifts this work would have been very difficult to achieve.

Thanks to my committee members, Drs. Gwyn Beattie, Larry Halverson and Gregory Phillips for serving on my committee.

Above all, thanks to my husband Sree and our daughter Aksheta for being very patient and for supporting my dreams throughout graduate school. Special thanks to my parents and my in-laws for making things easier for me with the new baby, and without whose support it would have been a far more difficult task.