A simple wax-embedding method for isolation of aphid hemolymph for detection of luteoviruses in the hemocoel

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Abstract

A protocol for isolating hemolymph from viruliferous aphids has been developed. This method uses warm melted wax to immobilize the aphid. Following removal of a hind leg, the hemolymph can be collected readily. Flushing with RNase-free water allows for collection of sufficient hemolymph for RNA extraction from individual aphids. The extracted RNA was successfully used for detection of barley yellow dwarf virus (BYDV) and pea enation mosaic virus (PEMV) from individual viruliferous Rhopalosiphum padi and Aceria thummi aphids, respectively. A TaqMan real-time RT-PCR protocol for quantitation of PEMV in the hemolymph of individual aphids was developed. The wax-embedding hemolymph collection technique provides a useful tool for studying molecular interactions between persistent and circulative plant viruses and their insect vectors.

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1. Introduction

Members of the Luteoviridae, such as barley yellow dwarf virus (BYDV), potato leaf roll virus (PLRV) and beet western yellows virus (BYYV) are crop pathogens of global economic importance (Sylvestre, 1989; Blackman, 2000). Luteoviruses are transmitted exclusively by aphids in a persistent or circulative manner. Circulative transmission of luteoviruses involves complex interactions between the virus and the aphid vector. During aphid feeding, the virions pass with the sap through the maxillary food canal of the aphid stylet into the gut. From the gut (midgut or hindgut) the virions penetrate the gut epithelium by endocytosis (Gildow, 1993; Gray, 1996). The virus-containing vesicle fuses with the plasma membrane, thereby releasing the virus, which readily passes through the basement membrane into the hemocoel. The virus is then transported to the accessory salivary gland. Upon aphid feeding, the luteovirus is injected into the plant with saliva via the salivary canal in the maxillary stylets. On passage through the aphid vector, the virus overcomes two membrane barriers (gut and accessory salivary gland) and may be protected from proteolytic degradation within the hemocoel by association with a GroEL-like protein called symbionin (van den Heuvel et al., 1997; Filichkin et al., 1997). The transmission of luteoviruses by aphids is influenced by multiple factors including the host plant, environmental conditions, as well as the aphid vector and virion surface structures (Gray and Gildow, 2003). On acquisition of a virus by an insect vector, transmission of the virus depends primarily on molecular interactions between the virions and the vector. These interactions determine whether or not the virus is transmitted, and the efficiency of transmission. For example, failure to transmit, or low efficiency of aphid transmission, can result from mutations that disrupt interaction between the viral capsid and the gut or accessory salivary gland membrane. Hence, movement of the virus from the gut to hemocoel or from the hemocoel to the accessory salivary gland may be blocked. Mutations in the viral capsid may also destabilize the virus, resulting in degradation of virions in the gut or in the hemocoel. Virus transmission may also be blocked by failure of virus release from the accessory salivary gland (Power and Gray, 1997; Gildow and Gray, 1993). To understand why an isolate is not transmissible, it is important to determine the stage at which virus transmission is blocked...
hemocoel or accessory salivary gland). Isolation of viral RNA from hemolymph and quantitation of virus by real-time RT-PCR can delineate the cause of attenuated virus transmission. Understanding the molecular interactions between luteoviruses and their aphid vectors may facilitate development of measures to prevent plant virus transmission by insect vectors.

Detection of plant viruses in their respective insect vectors is also an important component of integrated pest management for virus control (Boonham et al., 2002). Several methods for isolation and detection of plant viruses in insect vectors have been described. These methods are based on detection of viral genomic DNA or RNA by PCR or RT-PCR (Lopez-Moya et al., 1992; Mehta et al., 1994; Minafra and Hadidi, 1994; Olmos et al., 1990, 1997; Stevens et al., 1997; Naidu et al., 1998; Vercruysse et al., 2000), real-time RT-PCR (Boonham et al., 2002; Fabre et al., 2003), or on detection of either viral structural proteins by ELISA (Gera et al., 1978; Abouamousheh et al., 1990; Stevens et al., 1997) or non-structural proteins (Chomchan et al., 2002). Methods used for isolation and detection of luteoviruses in aphids have also been reported for BYDV (Rochow and Brakke, 1964; Lister and Rochow, 1979; Torrance, 1987; Robertson et al., 1991; Canning et al., 1996; Chay et al., 1996; Fabre et al., 2003), PLEV (Singh et al., 1995, 1996, 2004; Singh, 1998, 1999; Nie and Singh, 2001), BWYV (Veidt et al., 1992; Reinbold et al., 2001) and pea enation mosaic virus (PEMV) (French et al., 1973).

To understand the mechanisms of transmission (or lack thereof), it is important to distinguish virus in the hemocoel, from virus that remains only in the gut. Yet, in most examples above, the methods describe use of whole insects, rather than insect hemolymph for isolation and detection of viruses. One exception is Chay et al. (1996) who used a microinjector to inject RNAse-free water into the aphid hemocoel. Diluted hemolymph was then collected on removal of a hind-leg from the aphid. However, this approach is technically challenging and relatively little hemolymph can be collected. A simpler method for collection of hemolymph from individual aphids in sufficient quantities to allow extraction of viral RNA is required.

The goal of this study of the mechanisms of luteovirus transmission was to determine why some BYDV and PEMV mutants were not aphid transmissible. To this end, a reproducible method for isolation and detection of viruses in the aphid hemocoel was critical. In this paper a rapid and simple approach for isolating hemolymph from individual viruliferous aphids is described. The isolation method is reliable and robust. Sufficient hemolymph can be acquired for extraction of viral RNA for detection of luteoviruses in the hemocoel of individual aphids by using reverse transcription polymerase chain reaction (RT-PCR) and high throughput quantitative RT-PCR (QRT-PCR).

2. Materials and methods

2.1. Virus isolates

Two luteoviruses, barley yellow dwarf virus (BYDV) and pea enation mosaic virus (PEMV) were used in this study. An infectious BYDV cDNA clone, pPAV6-129 (Koev et al., 2002) was used for inoculation and aphid feeding. Infectious PEMV-1 and PEMV-2 clones (Dentler et al., 1997) were generously provided by Dr. G.A. de Zoeten. A non-transmissible PEMV mutant (RTDVY114N) was also used in all assays. The infectious Brome mosaic virus (BMV) clones (pT7B1, pT7B2 and pT7B3) were obtained from Dr. A.L.N. Rao, University of California at Riverside.

2.2. Aphids

The bird cherry-oat aphid, *Rhopalosipnum padi* and pea aphid, *Acyrthosiphon pisum* were reared on oat and pea, respectively (22–24 °C, L: D 12:12 h). The non-viruliferous aphids were given a 48 h acquisition period on BYDV, PEMV or BMV-infected plants before hemolymph was extracted. Total RNA was also extracted from whole aphids.

2.3. In vitro transcription of viral RNA and inoculation of viral transcripts

Methods used for in vitro transcription of PANV-129 and inoculation of the transcripts to oat protoplasts were as described previously (Dinesh-Kumar and Miller, 1993; Koev et al., 2002). The transcripts of PEMV and BMV genomes were synthesized by using the mMessage mMachine™ T7 kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Equal amounts (approximately 1 μg of RNA for each plant) of PEMV RNA-1 and RNA-2 were mixed and rubbed onto 7-day-old pea seedlings (*Pisum sativum* cv. Progress No. 9). A similar method was also used for inoculation of BMV; the mixed RNAs 1.2 and 3 were mechanically inoculated on to 7-day-old barley seedlings (cv. Manchuria).

2.4. Virus purification and aphid transmission of BYDV

The method used for semi-purification of BYDV from infected oat protoplasts was modified from Chay et al. (1996). Briefly, the infected protoplasts were harvested 48 h post inoculation by centrifugation at 700 × g for 5 min. The cells were resuspended in 300 μl of 10 mM sodium phosphate buffer pH 7.0, sonicated for 2–3 s, and centrifuged at 8000 × g for 5 min. The supernatants were then centrifuged at 50k rpm for 30 min (Sorval Discovery M150 centrifuge, S150AT rotor). The pellets were resuspended in 100 μl of 10 mM sodium phosphate buffer pH 7.0 and diluted 1:1 in 50% sucrose for feeding to virus-free *R. padi* through paraffin membranes. Following a 16–18 h acquisition period, 15–20 aphids were placed on individual oat seedlings (*Avena sativa* cv. Clinkton64). The aphids were later killed. The infected plants were used for aphid feeding tests. Hundred to 200 aphids were fed on the infected plants for 24–48 h before they are used for isolation of RNA.

2.5. Isolation of hemolymph

The first step is to immobilize the aphid on a thin layer of melted wax. Wax (Paraseal®, W & F products Inc.) was melted and dropped onto a piece of Parafilm® (American National
Can™) to produce a thin layer (~1–2 mm). The wax on the parafilm was then remelted on a warm hot plate (50-60 °C) and an aphid placed on to the melted wax. Once the melted wax completely covered the body of the aphid, the parafilm containing the embedded aphid was removed from the hot plate to allow the wax to harden around the aphid (Fig. 1A). Under a binocular microscope, a hind leg was removed from the embedded aphid using a needle (26 3/8 Gauge Beckin Dickinson) (Fig. 1B and C). RNase-free water was flushed on to the wound three or four times using a 10 μl pipette. The diluted hemolymph was then collected by using a Pasteur pipette (Fig. 1D). The hemolymph samples were then used for total RNA extraction for RT-PCR and qRT-PCR detection of viral RNA.

2.6. Extraction of total RNA from aphids

Total RNA was prepared by using a Stratasp**e Total RNA Microprep Kit (Stratagene®). Hemolymph isolated from the hemocoeal of individual or multiple *R. padi* or *A. pisum* was resuspended in 50 μl of the lysis buffer for individual aphid, or 100 μl for two to three aphids. Total RNA from whole aphids was extracted by grinding one aphid per 100 μl and two to three aphids in 200 μl of lysis buffer. The following extraction procedures were performed according to the manufacturer’s instructions.

2.7. RT-PCR and primers

The 1st strand viral cDNA was synthesized from the purified total RNA using SuperScript®II RNase H™ Reverse Transcriptase (Invitrogen®), according to the manufacturer’s directions. Briefly, 4 μl of RNA extraction, 2 μl of 2.5 mM dNTP mixture, 1 μl of 20 pmol reverse primers, and 5 μl of RNase-free water were mixed and incubated at 65 °C for 5 min; 4 μl of 5 × reaction buffer, 1 μl of RNasig (Promega) and 2 μl of 0.1 M DTT were then added into the tubes and incubated at 42 °C for 2 min before adding 1 μl of Super Script II reverse transcriptase. The reactions were carried out at 42 °C for 50 min and the reactions were stopped by incubating at 95 °C for 5 min. The cDNA was stored at −80 °C. PCR was subsequently performed in a 20 μl volume, which contained 2 μl of 1 strand viral cDNA, and 1 unit of Taq DNA polymerase (Takara). Amplification was carried out using a 1 min hot start of 94 °C, 40 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1.5 min and a final elongation step of 72 °C for 7 min. The primer used for 1st strand viral cDNA synthesis of PAV6-III was 5'-GGAAGGC CGGCATCC-3', which corresponds to the nucleotide (nt) positions 5681 to the 3' end of the viral RNA. RT-PCR of BYDV RNA was performed using the 3' end reverse transcription (RT) primer along with the 5' end primer: 5'-GCTCA GTGGAGGGATT (corresponding to nts 5096-5114 of BYDV RNA). For PEMV-1 RNA, the 3' end RT primer and 5' primer were: 5'-GAACCGATTGCAA TCTAGGG-3' (nts 3849-3870) and 5'-CCATATTGGAGGAAATGGC-3' (corresponding to nts 3726-3744), respectively. The 3' end RT primer used for BMV first strand cDNA synthesis was 5'-GGCGATTAAAGTGGGTAAGA-3' (nts 2227-2207) of BMV RNA 3. The 5' end PCR primer was 5'-GTTCAGACTCCCGAAGA-3' (nts 1638-1658).

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Fig. 1. Isolation of hemolymph from aphids. (A) Individual aphid was embedded in a thin layer of melted wax on a piece of parafilm. (B) A hind leg (indicated by arrow) was dissected to release hemolymph. (C) The hind leg was detached from the aphid body and hemolymph released on to the wax surface. One to 2 μl of RNase-free water was added to the hemolymph (arrow). (D) The diluted blood was collected using a pipette (arrow). (C) and (D) were repeated several times.
2.8. Quantitative RT-PCR (QRT-PCR)

Real-time RT-PCR was designed for quantitative detection of PEMV-1 viral RNA from the hemolymph of viruliferous *A. pismus*. The primers used for QRT-PCR were the same as those used for routine RT-PCR. A TaqMan probe, 5′-ACATGTCTTATAGGACCCTCTGG-3′ (nts 3771–3792), was designed within the conserved ORF2 region of PEMV RNA-1. The 5′ end of the probe was labeled with 6-FAM (synthesized by IDT) and the 3′ end was labeled with the Blackhole Quencher I (synthesized by IDT). A pair of primers for *A. pismus* 18S rRNA was also designed and used as a control for RNA samples: 5′-CTGGTTGATCCTGCCAGTAG-3′ (18S RNA nts 4–23) and 5′-GTCGGAATCCTGCACGGC-3′ (18S nts 190–174). PCR products were detected using Sybr-Green labeling (Molecular Probes, Whitham et al., 2003) for quantitative detection of 18S rRNA.

The QRT-PCR was performed in two steps. The RT reaction was carried out as described in Section 2.7. TaqMan reactions were set up in 96-well reaction plates. Each reaction (25 μL) contained 2 μL of the 1st strand DNA RT synthesis, 7.5 pmol each of the forward and reverse primers, 5 pmol of 6-FAM probe, 0.3 mM dTTP, 3 mM MgCl2 and 1 unit Platinum® Taq DNA polymerase (Invitrogen). For the control QRT-PCR of 18S rRNA, 100 pmol of Sybr-Green and 10 nM of fluorescent Calibration Dye were added into the 25 μl reactions (Whitham et al., 2003). PCR reactions were performed on a BIO-RAD iCycler™ IQ system using the thermal cycle conditions: 95 °C for 1 min, followed by 94 °C for 45 s, 58 °C for 1 min and 72 °C for 1 min for 40 cycles. A standard curve consisting of serial 1:10 dilutions was prepared with cDNA concentrations of 100, 10, 1, 0.1, and 0.01 ng/μL. PCR reactions were performed in triplicate and analyzed on a BIO-RAD iCycler™ IQ Optical system using Software Version 3.0a.

3. Results

3.1. Wax-embedding technique for isolation of aphid hemolymph

 Ideally, isolation of aphid hemolymph from the hemocoel should provide sufficient viral RNA from an individual aphid for quantitative detection of virus. However isolation of hemolymph uncontaminated by gut contents presents a number of technical challenges. It is difficult to remove an aphid leg and inject water to collect hemolymph without damaging the aphid to the extent that hemolymph becomes contaminated with other aphid fluids or tissues. To overcome this problem, Aphids were immobilized in melted wax. As the wax cools and hardens, a hind leg was removed from the embedded aphid using a needle (26 3/8 Gauge Beckin Dickinson) (Fig. 1B and C). RNase-free water was flushed on to the wound three or four times using a 10 μL pipette. The diluted hemolymph was then collected by using a Pasteur pipette (Fig. 1D). Details of this technique are described in Section 2. Two luteoviruses, BYDV and PEMV, and their respective vectors *R. padi* and *A. pismus* were used to evaluate the versatility of this method.

Fig. 2. RT-PCR detection of BYDV in the hemolymph of individual *R. padi*. M: mock; T: total RNA sample extracted from the whole body of a single aphid. H1–H4: RNA samples extracted from the hemolymph of individual *R. padi*. BYDV RNA was detected from all of the viruliferous aphids, but not from the control aphid (M).
the aphid vector, PEMV and its vector *A. pisiurn* were used to perform RT-PCR and QRT-PCR. RNA extracted from hemolymph samples from viruliferous *A. pisiurn* individuals as well as from whole aphids was used for RT and QRT-PCR experiments. RNAs of aphid transmissible PEMV (AT+) and a non-transmissible mutant, RNA1RTDY114N (AT−) (Li, unpublished result) were used for the RT-PCR assay. Both PEMV AT+ and AT− were detected in hemolymph samples of single viruliferous *A. pisiurn* aphids (Fig. 4). The QRT-PCR showed that both AT+ and AT− viruses had similar viral RNA concentrations in the aphid hemolymph (Fig. 5), 30 min after feeding on infected plants for 48 h. These results suggest that the AT− virus entered the hemocoel and attained titers comparable to those of wild type PEMV. This result also indicates that the failure of aphid transmission of RNA1RTDY114N is not due to blockage of virus entry into the hemocoel. This result illustrates the importance of this method for detection of luteoviruses in the hemocoel, for understanding the stage at which virus transmission is blocked in the aphid.

4. Discussion

A particular concern in isolating hemolymph from a viruliferous aphid vector is the risk of cross-contamination from the aphid gut, honeydew or styles during the hemolymph collection process. Cross-contamination was shown not to occur, because BMV was not detected in hemolymph extracts even though it was easily detected in whole aphids (Fig. 3). The lower amount of BMV than BYDV in whole aphids, as detected by QRT-PCR, may be because the aphid is not a vector of BMV. BMV may degrade in the aphid gut. These results resemble those of Chay et al. (1996).

The advantages of the wax-embedding technique compared to the published hemolymph isolation method (Chay et al., 1996) are: (1) the aphid is immobilized, which expedites collection of hemolymph; (2) cross-contamination from the styles and the other parts of the body is reduced because the aphid is submerged in wax; (3) hemolymph collection is conducted outside of the aphid body on the surface of the wax, which further alleviates the possibility of gut cavity contamination; (4) the technique is simple, reliable and robust.

Methods have been reported for isolation of viruses from whole aphids for detection by ELISA (Lister and Rochow, 1979). The wax-embedding technique may also be applied for detection of coat proteins or capsids from the hemocoel of viruliferous aphids.

In summary, the wax-embedding method for hemolymph collection will facilitate research on the molecular interactions between luteoviruses and their aphid vectors. This method will be used to clarify the roles of virus structural proteins in virus transmission via the aphid vector, and will provide the means to identify capsid protein motifs involved in movement of the virus within the aphid. This technique can also be applied for study of interactions between other persistent and circulative viruses and their respective insect vectors, and for investigation of passage of West Nile virus from the mosquito to avian hosts. This procedure has also been modified for isolation of hemolymph from adult *Drosophila melanogaster*. In this case, removal of a wing...
from the wax-embedded vinegar fly provides more hemolymph than tested with a leaf (unpublished result).

Transmission of luteoviruses is dependent on multiple factors. Identification of the precise mechanisms associated with reduced virus transmission will facilitate understanding of the molecular basis of aphid transmission. A sensitive method for the detection of ingested viruses in the aphid hemolymph is particularly important for elucidation of virus transmission determinants. In this paper a relatively easy and cost effective approach for isolating hemolymph and luteovirus RNA from the aphid hemolymph was described. This novel and reproducible method provides sufficient viral RNA for detection of viruses in the hemolymph of both small and large, single aphids (R. padi and A. pismum, respectively) by RT and QRT-PCR. This method could easily be used to isolate hemolymph from other important insect vectors of plant viruses, such as whiteflies, leafhoppers, planthoppers and mealybugs.

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