Citrus psorosis virus: nucleotide sequencing of the coat protein gene and detection by hybridization and RT–PCR

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Introduction

A bark scaling disease of citrus was first observed in Florida and California in the 1890s. Fawcett (1933) observed a mosaic in young leaves of citrus trees with bark scaling symptoms and suggested the disease was caused by a virus, which he named citrus psorosis virus (CPV). Another bark scaling disease of citrus was described as citrus ringspot virus (CRSV) by Wallace & Drake (1968). The leaf and bark symptoms caused by CPV and CRSV are similar, but they have often been considered to be different diseases based on symptom severity. It has been previously suggested that the various isolates of CRSV and CPV are either identical or are biologically and serologically diverse strains of the same virus (da Graça et al., 1991; Garcia et al., 1993, Navas-Castillo et al., 1993; Navas-Castillo & Moreno, 1993). Therefore, we refer to all isolates of CRSV and CPV as CPV.

CPV is a multicomponent ssRNA virus with a coat protein of approximately 48 kDa (Derrick et al., 1988a, b; 1991; Garcia et al., 1991). The ssRNA viral genome is contained in short (300–500 nm) and long (1500–2500 nm) filamentous particles that are readily separated by sucrose density-gradient centrifugation. A dsRNA component that co-sediments with long particles and may be required for infectivity is also associated with CPV (Derrick et al., 1991). CPV particles appear as spiral filaments approximately 10 nm in diameter when viewed by electron microscopy after positive staining and are referred to as spiroviruses (SV) (Derrick et al., 1993). In contrast, 3–4 nm diameter particles were observed in electron microscope examinations of negatively stained CPV preparations (Garcia et al. 1994; Milne et al., 1997). The authors stated that these particles with novel filamentous morphology are ‘collapsed double-stranded forms of nucleocapsid-like, highly flexous open circles’. They further suggested that the 10 nm diameter linear, spiral particles are a somewhat misleading form of a more fundamental particle. We suggest
that the 10 nm diameter particles, which are detected by positive staining, may be the native form, and that the 3–4 nm particles are produced by the high ionic concentrations associated with negative staining, which could disrupt the native particles.

We report here the nucleotide sequence of the coat protein gene (CPG) of an isolate of CPV and the development of hybridization and RT–PCR based detection methods for CPV. Moreover, we show that short particle RNA of CPV contains negative sense RNA, and that the CPG is encoded by short particle RNA but not by long particle RNA or sedimentable dsRNA.

Methods

- **Virus isolates.** Florida virus isolates CPV-4 and CPV-6 (formerly referred to as CRSV-4e and CRSV-6b1) were biochemically characterized and isolated from single lesions on *Chenopodium quinoa* Willd. (Garnsey & Timmer, 1980, 1988). Isolates F113 and F123 were collected from citrus trees with bark scaling symptoms in Florida and propagated by graft inoculations. All isolates were maintained in sweet orange (*Citrus sinensis* (L.) Osbeck., cv. Madam Vionus) and/or grapefruit (*C. paradisi* Macf., cv. Duncan) seedlings under greenhouse conditions at Lake Alfred, Florida. All other CPV isolates used in this study were obtained from the USDA-ARS quarantine facility at Beltsville, Maryland, USA.

- **Cloning and sequencing.** Virus particles were purified from young symptomatic leaves containing isolate CPV-4 as described by Derrick et al. (1991). Following final purification of top component virus particles by agarose gel electrophoresis, RNA was isolated as previously described (Derrick et al., 1991). cDNA was synthesized and ligated into Lambda ZAP Express (Stratagene) according to the manufacturer’s protocol with some modifications. Briefly, cDNA synthesized using random primers was blunt-ended, and ligated to EcolI adapters. The cDNA was fractionated on a Sephacryl S-400 spin column, ligated to digested ZAP Express, packaged and plated on *E. coli* XL-1 Blue MRF’ cells.

  The resulting library was immunoscreened (Stratagene picoblue protocol) using a monoclonal antibody to isolate CPV-4 capsid protein (MAb-4; Derrick et al., 1993) preabsorbed with an *E. coli* cellular lysate (Sambrook et al., 1989). Selected clones were excised using the ExAssist/XLOR system (Stratagene) to yield pBK phagemids. Plasmid DNA was isolated (Nicoletti & Condorelli, 1993) and sequenced in both directions by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida. The sequences were analysed using the computer programs Sequaid II version 3.81 (Rhodes & Roufa, 1985) and PC GENE release 6.8 (IntelliGenetics). All sequences were compared with the NCBI database using the BLAST program of the University of Wisconsin Genetics Computer Group, version 8.0 (Altschul et al., 1990).

- **Northern blot and RNA:RNA hybridization assays for CPV CPG.** Full-length 32P-labelled riboprobes of the CPG were prepared using *in vitro* transcription (MAXiscript, Ambion). For Northern hybridizations, RNA was extracted from individual sucrose density-gradient fractions (Derrick et al., 1991) and subjected to electrophoresis on 0.8% agarose denaturing gels containing 0.5% formaldehyde (Sambrook et al., 1989). The gels were blotted using a Turboblotter (Schleicher and Schuell) to nylon membranes (Magnagraph, MSI) in 3 M NaCl, 8 mM NaOH and 2 mM Sarkosyl for 2 h (Chomczynski, 1992).

Total nucleic acid extracts for dot-blot hybridizations were prepared by mixing 300 mg of soft leaf tissue in a 2 ml microcentrifuge tube with 1.5 ml 100 mM Tris–HCl pH 8.0, 2% SDS and two 1/4 inch steel pellets and shaking for 1 min in a Mini-bead Beater model 8 (Biospec Products). The extracts were clarified by centrifugation and 500 µl was transferred to a fresh tube and extracted with an equal volume of phenol–chloroform (1:1). The aqueous phase was precipitated with ethanol, and the pellets resuspended in 100 µl of 2 × SSC containing 10% formaldehyde. The samples were heated at 65 °C for 10 min, chilled on ice and 2 µl was spotted onto nylon membranes (MSI).

For tissue print hybridizations, young shoots were cross-sectioned with a razor blade and pressed onto nylon membranes. Nucleic acids were cross-linked to the membranes using a Stratagene Stratalink. Pre-hybridizations and hybridizations were done in 5 × SSC containing 50% formamide, 2 × Denhardt’s solution, 1% SDS, and 0.1 mg/ml salmon sperm DNA. After overnight hybridization at temperatures ranging from 55 to 66 °C the membranes were washed twice at room temperature in 2 × SSC containing 0.5% SDS and twice in 0.1 × SSC, 0.5% SDS at the temperature used for hybridization. Membranes were exposed to X-ray film (X-Omat, Kodak) and the results digitized using an Alpha Innotech IS 1000 Digital Imaging System.

- **RT–PCR of the CPG of CPV.** Primers CPV1 (5’ GCTTCTCG-GAAAGCTGATG) and CPV2 (5’ TCGTTTTTGTCAACACACTCC) were designed for the amplification of a 600 bp product from nt 654 to 1253 of the CPG using Primer version 0.5 (Whitehead Institute for Biomedical Science). Primers CPV3 (5’ CCTAAAGCTACAGATGC-ATCTCTATTTAG) and CPV4 (5’ GACCGATTCTAAAGGACT-AACATGCAAGC), containing additional Nhel and EcoRI restriction sites respectively (underlined), were used to amplify a 1365 bp region containing the complete ORF of the CPG. RNA for RT–PCR with primers CPV1 and CPV2 was prepared by grinding 0.5–1 g of young leaf tissue in 50 mM Tris pH 8.0, 2% SDS and 0.5% 2-mercaptoethanol, extracting with phenol–chloroform (1:1) and precipitating with ethanol. The pellets were resuspended in 40 µl of RNase-free water. For RT–PCR with primers CPV3 and CPV4, 1 µl of young leaves was extracted and subjected to one cycle of differential centrifugation prior to preparation of RNA (Derrick et al., 1991).

  For cDNA synthesis, 4.75 µl of RNA was added to 1.25 µl each of forward and reverse primer (10 µM stock), denatured at 65 °C for 5 min and chilled on ice. AMV-RT buffer (2.5 µl) (Promega), 1.25 µl of 2 mM dNTPs, 1 µl RNasin (40 U/µl) and 0.5 µl AMV-RT (10 U/µl) were added and the samples were incubated for 1 h at 42 °C. cDNA (0.5 µl) was added to 49.5 µl of a PCR cocktail containing 1 × PCR buffer (Idaho Technologies), 3 mM MgCl₂, 200 µM dNTPs, 0.5 μM of each primer and 1 unit of Taq polymerase. Amplification began at 94 °C for 1 min followed by 35 cycles of: 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, with a final extension of 72 °C for 5 min. PCR products were fractionated on 8% polyacrylamide gels, stained with ethidium bromide and viewed over a UV light source. These images were digitized using an Alpha Innotech IS 1000 Digital Imaging System.

- **Expression of CPV CPG in E. coli.** A clone containing the full-length CPG of CPV-4 was amplified by PCR using primers CPV3 and CPV4. The PCR product was digested, repurified and ligated into Nhel and EcoRI sites of the pETH 3a expression vector (McCarty et al., 1991), a modified pET vector (Novagen). The resulting plasmid was used to transform *E. coli* BL21(DE3) pLYSs cells (Novagen). For expression, single colonies were grown in the presence of ampicillin (200 µg/ml) and chloramphenicol (34 µg/ml) to an OD₆₀₀ of 0.6 and induced with 0.4 mM IPTG (Novagen protocol). Aliquots of induced cells were
concentrated by centrifugation, disrupted by boiling in gel loading buffer (Laemmli, 1970) and assayed by Western blotting using MAb-4.

Results and Discussion

Cloning, sequencing and expression of the CPG of CPV

One of our research objectives was to clone and sequence the CPG of CPV, which we believed to occur on the short particle RNA. To accomplish this a cDNA library of short particle RNA was produced in Lambda Zap Express and screened with a monoclonal antibody to the coat protein. Twelve immunopositive plaques were obtained and excised as pBK phagemids. Nucleotide sequencing of three of these phagemids revealed a 1329 nt ORF with an ATG start codon and ending with two closely spaced in-frame termination codons (Fig. 1). This ORF encoded a 49 kDa protein composed of 439 amino acids. There were no ORFs of significant size on the complementary strand.

Expression of the CPG ORF in E. coli BL21(DE3) pLysS, a host for the overexpression of recombinant proteins, gave a single immunoreactive peptide similar in size to the coat protein of CPV-4 (Fig. 2). Previous evidence that the 48 kDa protein isolated from CPV-infected tissue is the coat protein includes: (1) the protein was consistently found in infectious fractions of purified virus preparations and (2) polyclonal and monoclonal antibodies to CPV reacted with the protein in Western blots and with virus particles in serologically specific electron microscopy (da Graça et al., 1991; Derrick et al., 1988b, 1991, 1993).

Detection methods for CPV

There is a need for sensitive non-biological methods of detecting CPV, which is one of the few remaining important graft transmissible disease agents of citrus that can only be detected by biological indexing. This indexing requires a temperature controlled greenhouse facility and 2–4 months for the evaluation of the indexed material. Even under optimum conditions, many isolates of CPV give faint, transitory leaf symptoms on indicator plants making them difficult to detect biologically. In order to provide a more rapid and effective detection system we have used the CPG sequence of CPV-4 to develop RT–PCR, hybridization and serological assays for CPV. Assays for CPV based on RT–PCR of a fragment of long particle RNA and ELISA using a polyclonal antibody to purified virus were recently reported (Garcia et al., 1997a, b).

For the RT–PCR based detection assay, primers (CPV1 and CPV2) were designed to amplify a 600 bp product between nucleotides 654 and 1253 of the CPG. These primers amplified a product from total nucleic acid preparations of various isolates of psorosis A, psorosis B and citrus ringspot from geographically diverse locations (Fig. 3). The PCR products showed minor differences in the migration rate when fractionated on a polyacrylamide gel which is probably due to the expected sequence differences in these isolates. These differences in migration rate are not seen when samples are fractionated on agarose gels (data not shown). Samples from uninfected trees and one from a tree affected with impietratura, which is caused by an unknown graft-transmissible agent (Roistacher, 1991), were negative (Fig. 3, lanes 2 and 8 respectively). This assay has also been used to detect CPV in infected field trees (data not shown).

For RT–PCR amplification of the complete ORF of the CPG gene, a primer pair (CPV3 and CPV4) was designed and used to amplify the CPG of several CPV isolates (Fig. 4). The sequence of the CPG of various isolates can now be determined and their expressed coat protein can be used as an immunogen. Preliminary evaluations of a polyclonal antiserum produced to expressed coat protein of CPV-4 have shown it to be useful for detecting a variety of CPV isolates.

Development of serological assays to detect all isolates of CPV may require making antibodies to several different isolates. Previous efforts to develop serological assays for CPV had limited success due to difficulties in the purification of virions for antisera production, and the considerable diversity of isolates. A polyclonal antiserum to CPV-4 was made that reacts to only a few isolates (da Graça et al., 1991) and an effort to produce monoclonal antibodies for broad spectrum assays of CPV isolates resulted in antibodies that react only with CPV-4 (Derrick et al., 1993). These monoclonal antibodies proved useful in this study for cDNA library screening but are of no value as a diagnostic reagent for CPV.

For a hybridization based assay for CPV, full-length 32P-labelled positive and negative sense riboprobes were prepared from linearized clones of the CPG. Positive sense probes hybridized to several isolates in tissue print and dot-blots at 55 °C (Fig. 5). At this temperature, non-homologous isolates could be detected without any appreciable hybridization to host RNA. At hybridization temperatures at or above 60 °C only the homologous isolate CPV-4 was detected (data not shown). CPV was detected in samples diluted to 1:625, in which the spotted sample contained total nucleic acids from the equivalent of 10 mg of tissue. Some isolates of CPV that could be detected by RT–PCR could not be detected by hybridization assays. This could be due to low titre of the virus. Efforts to detect these isolates at hybridization temperatures lower than 55 °C were not successful due to non-specific reactions with host nucleic acids.

Northern blot analysis using positive and negative strand CPG riboprobes

To confirm that short particle RNA contained the CPG and to determine if the gene is also present on the long particle RNA and/or sedimentable dsRNA, purified RNA preparations were analysed by Northern blotting. Short and long particles and sedimentable dsRNA of CPV-4 were separated by sucrose density-gradient fractionation, and fractions containing virus...
Fig. 1. Nucleotide and deduced amino acid sequence of the cloned CPG of CPV-4 and partial CPG sequence of CPV-6. The CPV-6 sequence was generated by cloning a 600 bp RT–PCR product from nt 685 to 1243 using primers CPV1 and CPV2 (underlined). Positions having identical nucleotides or amino acids are marked with a dot. Stop codons for CPV-4 are marked with asterisks.
Fig. 2. Western blot analysis of CPV coat protein expressed in *E. coli*. Proteins were extracted from healthy leaves (lane 1), CPV-4 infected leaves (lane 2), *E. coli* transformed with vector alone (lane 3) and *E. coli* expressing the CPG of CPV-4 (lane 4). Blots were developed using a monoclonal antibody (MAb-4) against CPV-4 coat protein.

Particles were identified by immunoblotting with MAb-4 (Fig. 6a). RNA from fractions 5–9, which contain the short particles, hybridized to a positive sense CPG riboprobe. RNA from fractions 10–13, which contain long particles and dsRNA, did not hybridize to a positive sense probe (Fig. 6b). No hybridization was observed with healthy citrus. These observations confirm that the CPG of CPV is on the short particle RNA only. The observation that the CPG is not repeated on long particle RNA or on the sedimentable dsRNA is consistent with reports that the short particle is required for infectivity (Derrick *et al.*, 1988). Using the same Northern protocol no hybridization was observed when probed with a negative sense transcript (data not shown). This indicates that the short particle contains negative sense RNA. Previous reports that nucleic acids isolated from highly infectious preparations of CPV-4 are noninfectious led to the suggestion that the viral genome was negative sense RNA (Derrick *et al.*, 1991; Garcia

Fig. 3. PAGE of RT–PCR products amplified from leaf extracts of various CPV isolates using primers CPV1 and CPV2. Lane 1, no template; 2, healthy citrus; 3, Florida CPV-4; 4, Florida CPV-6; 5, Argentina B75; 6, Florida F113; 7, Spain B96; 8, Spain impietratura B103; 9, Florida F123; 10, Argentina B84; 11, a clone of CPV-4 CPG. Arrow indicates the location of the amplification product. DNA markers are indicated on the right.

Fig. 4. PAGE of RT–PCR products of the CPG of CPV amplified using primers CPV3 and CPV4. Lane 1, healthy citrus; 2, Florida CPV-4; 3, Florida CPV-6; 4, Spain B99; 5, a clone of CPV-4 CPG. Arrow indicates the location of the amplification product. DNA markers are indicated on the right.

Fig. 5. Detection of various isolates of CPV by hybridization with a positive strand CPG riboprobe. Samples were (a) cut tissue blotted directly to nylon membranes or (b) total nucleic acid leaf extracts. For nucleic acid extraction leaves were extracted as described, denatured in formaldehyde–SSC and spotted. Samples were: healthy citrus (H), Florida CPV-4, Florida CPV-6, Argentina B75 and Spain B96.

Fig. 6. Analysis of sucrose density-gradient fractions of CPV-4 by (a) immunoblotting with MAb to CPV-4 and (b) Northern blotting and hybridization with a positive strand CPG riboprobe. RNA markers are indicated on the right of the Northern blot.
et al., 1991). Perhaps the viral replicate, required for negative sense viruses, is part of a labile replication complex associated with the dsRNA that co-sediments with the long particles. A loss of infectivity of CPV-4 has been associated with the failure to detect the sedimentable dsRNA in preparations kept at 4 °C for 24 h (Derrick et al., 1991). The virus particles containing ssRNA appear more stable, and can be isolated from preparations kept at 4 °C for several days. This suggests that the sedimentable dsRNA is not the result of hybridization of ssRNAs of opposite polarities following removal of the coat protein.

Sequence analysis of the CPG of CPV

A 600 nt fragment from RT–PCR of isolate CPV-6 was cloned and sequenced for comparison with CPV-4 (Fig. 1). The sequence of isolate CPV-6 has 86% (nucleotide) and 96% (amino acid) identity to CPV-4. BLAST analysis of the sequences of the two isolates did not reveal any similarity to known viral sequences. This is consistent with previous suggestions (Derrick et al., 1988b, 1993) that SV represent a new group of plant viruses.

The diversity in the nucleotide sequences of isolates CPV-4 and CPV-6 and the apparent failure of some isolates to hybridize with riboprobes to the CPG of CPV-4 is consistent with the biological and serological diversity reported for CPV isolates. An example of the diversity among isolates of CPV is shown in comparisons of CPV-6 and CPV-4, which are only weakly related serologically (Derrick et al., 1993). Moreover, CPV-6 causes severe bark scaling on citrus, while CPV-4, which was isolated from a tree with leaf symptoms, has never been observed to cause bark scaling. Also, leaf symptoms associated with CPV isolates vary from severe to mild with some isolates causing no leaf symptoms (Passos, 1965). CPV-4 will probably be considered the type strain of CPV, even though it does not cause bark scaling.

The successful cloning, sequencing and bacterial expression of the CPG of CPV-4 along with the recent work by Garcia et al. (1997a, b) will provide tools for studies on the replication strategies of SV. Moreover, additional sequence comparisons among the various diverse isolates can be expected to lead to better non-biological assays for CPV by RT–PCR, hybridization, or by serology using expressed coat protein from different isolates as an immunogen for antibody production.

Since CPV has a wide experimental host range (Timmer et al., 1978), it could be expected that CPV and possibly other SV will be found in natural infections of plants other than citrus. There are recent reports of viruses with some similarities to SV infecting ranunculus (Vaira et al., 1997) and tulip (Morikawa et al., 1995).

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References


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