Detection of Citrus Ringspot Virus and Citrus Psorosis Associated Virus Using PCR

M. L. García, M. E. Sánchez de la Torre, N. Costa, and O. Grau

ABSTRACT. The citrus ringspot isolate CtRSV-4 was purified, and the dsRNA associated with the bottom component in a sucrose gradient was reverse transcribed and cloned. One of the clones (224m) was partially sequenced and, from this sequence, a set of primers encompassing a 218 bp fragment was designed. These primers were used for reverse transcription and amplification by the polymerase chain reaction (RT-PCR) of crude extracts from healthy or infected citrus tissue. A 218 bp DNA segment was obtained from plants infected with ringspot CtRSV-4 or with psorosis CPsAV-90-1-1 but not from healthy plants, indicating that both viruses were specifically detected in infected citrus.

Psorosis is one of most widespread citrus diseases and the cause of important losses in many countries (11). In most countries the disease can be easily avoided in new plantings by using certified virus-free budwood, but in Argentina, where a severe form of the disease is present, it seems to be naturally spread by an unknown vector, thus, causing continuous losses in the Mesopotamian region. Presently, reliable diagnosis of the disease can be achieved only by biological indexing on indicator plants grown in a temperature-controlled greenhouse (9). This procedure is slow and expensive and it does not allow large-scale indexing, as required to control disease spread through nursery plants or by natural vectors. In addition, some isolates can be missed if the greenhouse does not provide the proper temperature conditions (9). Quick and reliable diagnostic procedures are urgently needed to control psorosis spread in Argentina.

A two-component virus with flexuous particles of unusual morphology, and a capsid protein about 48 kDa, has been associated with different isolates of citrus ringspot (CtRSV) and psorosis (2, 5, 6, 8). Low titer and unstability of this virus has hindered acquisition of specific antibodies for serological diagnosis. An antiserum to the 48 kDa protein of the isolate CtRSV-4 (1) reacted in western blots with the protein associated with other isolates of CtRSV or the citrus psorosis associated virus (CPsAV) (1, 5, 8), but this antiserum does not allow quick detection of these diseases in plant extracts (8). Recently, new antibodies prepared to a psorosis isolate reacted with infected extracts in ELISA (unpublished results), but reactivity of this antiserum with isolates from different origins is yet unknown. A sensitive technique like reverse transcription polymerase chain reaction (RT-PCR) might be very convenient for diagnostic purposes, but no sequence of the virus genome was yet available.

CtRSV-4 and CPsAV-90-1-1 are closely related and they are considered as viruses of the same group or different strains of the same virus (5, 6, 8), but, so for, only the isolate CtRSV-4 from Florida has been successfully purified and detectable amounts of single and double stranded RNA, (ss and dsRNA) obtained (3). We have obtained complementary DNA (cDNA) clones from CtRSV-4 dsRNA, sequenced them, and prepared primers to set a RT-PCR system for detection of CpsAV. In this paper, we present preliminary results indicating that CPsAV can be detected in crude extracts from graft-inoculated citrus seedlings.

CtRSV-4 was described in Texas, U.S.A. (7) and it was kindly provided by Drs. L.W. Timmer and K. Derrick. Citrus psorosis associated virus isolate 90-1-1 is from Concordia, Entre Rios, Argentina. Virions were purified and the bottom component separated as described by Derrick et al. (2), using *Chenopodium quinoa* local lesions as starting material. Total nucleic acids were extracted from bottom-sucrose gradient fractions further purified by agarose gel electrophoresis. Bottom-dsRNA was extracted from the agarose gel by the RNAid kit (BIO 101, CA., U.S.A.) and used for cloning.

The crude extracts used for RT-PCR assays were obtained by grinding healthy and infected young shoots in Eppendorf tubes using minipestles and 10 ml/g of homogenization buffer (0.05 M Tris-HCl, pH 8, 0.1% ascorbic acid, 0.1% cysteine and 0.5% 2-mercaptoethanol). The homogenates were centrifuged and the pellet discarded. Total nucleic acids were phenol extracted from the supernatant after treatment with 0.2 mg/ml proteinase K and 1% SDS for 1 h at 42°C. After ethanol precipitation, the pellets were resuspend in RNAse-free water.

Complementary DNA was obtained by reverse transcription using the bottom-dsRNA and random primers. The cDNA was prepared using the Librarian II kit (Invitrogen Corp., CA., USA) according to the manufacturers instructions. The recombinant plasmids were used to transform electrocompetent *E. coli* LE392 by electroporation. The library was screened with a ^{32}P-labeled cDNA probe prepared from the bottom-dsRNA by "short copy" (10). To discard clones containing a cDNA insert from cellular RNAs, labeled cDNA prepared from healthy citrus RNA was used as probe in a second screening. One of the selected clones, which is 1,200 bases long was called 224m. The viral origin of this clone was confirmed by Northern blot hybridization of bottom-dsRNA with the insert of the 224m clone as probe. The clone was then partially sequenced and from this sequence two primers were designed.

Nucleic acids obtained from 100 mg of tissue were heat denatured at 100°C for 3 min, and a 1/10 aliquot was reverse transcribed using the two primers designed and Retrotherm TMRT (Thermostable Reverse transcriptase and DNA polymerase, Epicentre Tecnologies), according to the manufacturers protocol. An aliquot of cDNA was amplified by PCR (4) using the same pair of primers. The PCR products were analyzed by agarose gel electrophoresis.

Figure 1 shows the PCR products obtained from clone 224m and from nucleic acid extracts from healthy and infected citrus tissue. Clone 224m yielded a 218 bp DNA fragment as expected from its sequence. A DNA fragment of the same size was also amplified from CtRSV infected but not from healthy tissue extracts, indicating that CtRSV can be specifically detected in infected

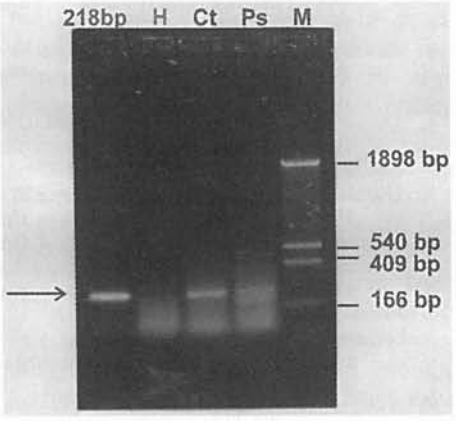

Fig. 1. Agarose gel electrophoresis of the PCR products obtained using the clone 224m (lane 218 bp), or total nucleic acid extracts from citrus tissue healthy (lane H), infected with CtRSV (lane Ct), or with CPsAV (lane Ps). Lane M: Molecular weight marker (Dde I digested pcDNAII DNA). Arrow indicates the expected 218 bp DNA fragment.

citrus leaves. The viral origin of the amplified band was confirmed by Southern blot hybridization (data not shown). CPsAV was also detected in total nucleic acid extracts using the same primers designed for CtRSV. This agrees with previous results (4) indicating that both virus are closely related. The band obtained from CPsAV-cDNA was weaker than that amplified from CtRSV infected tissue. This difference might reflect titer variations in tissues infected by both isolates. In fact, the infectivity on *C. quinoa* and the amount of coat protein present in infected tissues is about 100 times lower for CPsAV than for CtRSV. Alternatively, it might be due to the presence of different sequence variants within CPsAV, some of which would not be amplified using the primers designed from the CtRSV sequence. To obviate this possibility, RT-PCR will be optimized for CPsAV using specific primers designed from its own sequence.

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