Characterization of Citrus Ringspot Virus

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ABSTRACT. The virus associated with citrus ringspot (CRSV) was further characterized. Nucleic acids associated with the short and long filamentous particles were isolated and appeared to be single-stranded RNA. Nucleic acid preparations of crude extracts or partially purified preparations were not infectious when assayed on *Chenopodium quinoa*. A sedimentable double-stranded RNA was associated with CRSV infections in *C. quinoa* and citrus. An antiserum, prepared to CRSV purified by sucrose gradient centrifugation and agarose gel electrophoresis, was used to detect the virus by serologically specific electron microscopy (SSEM) and on nitrocellulose blots of agarose electrophoresis gels.
Index words. citrus psorosis.

A disease of citrus that causes bark scaling was first described and named citrus psorosis by Swingle and Webber in 1896. In 1933 Fawcett (4) observed a mosaic in young leaves of trees with psorosis and suggested the disease was caused by a virus. Two forms of psorosis, A and B, have been described (1), and a flexuous rod-shaped particle has recently been associated with psorosis (6). Citrus ringspot virus (CRSV) was first described by Wallace in 1968 (9). The leaf and bark symptoms caused by psorosis A and B and CRSV are somewhat similar, but they have been considered to be different diseases because psorosis A produces only mild flecking in young leaves whereas CRSV and psorosis B produce spots, ringspots and neurotic lines which persist in mature leaves. In contrast, cross protection tests with various isolates tend to indicate that psorosis B is a severe form of psorosis A and the CRSV is similar, if not identical, to psorosis B (8). This view is supported by our recent findings, and at this point it would appear that various isolates of CRSV and citrus psorosis virus are either identical or strains of the same virus. Thus, we consider ringspot and psorosis to be synonymous.

Prior to the use of virus-free budwood, psorosis was one of the most important diseases of citrus in the world. In Argentina, where psorosis appears to be spread by an unknown aerial vector (7), or where certified budwood programs are not used it remains a major problem. Since the time psorosis was described as the first virus disease of citrus, a number of other citrus viruses have been described and characterized. However, despite improvements in methods for purifying and characterizing viruses the actual identity of the causal agent of psorosis has, until recently, remained an enigma.

The infectivity associated with CRSV was readily separated into top (TC) and bottom (BC) components by sucrose density gradient centrifugation (SDGC) (2, 3). Short and long very flexible filamentous particles were associated with TC and BC, respectively. A 48-kilodalton (Kd) protein, which may function as a capsid protein, was associated with the particles. We now report some additional characteristics of this unusual virus.

MATERIALS AND METHODS

Virus isolate and hosts. The CRSV isolate used in this study was CRSV-4 that had been through several successive single lesion transfers on *C. quinoa* followed by transfer to *Gomphrena globosa* and subsequently to Duncan grapefruit (5).

Partial purification. Leaf tissue was pulverized with liquid nitrogen.

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The resulting frozen powder was transferred to a mortar and pestle at room temperature and ground with 7 ml of 0.05 M Tris:HCl (pH 8.0), 0.1% ascorbic acid, 0.1% L-cysteine, and 0.5% 2-mercaptoethanol (TACM) per gram of tissue. All subsequent purification steps were done at 4 C. The extracts were filtered through cheesecloth and subjected to one cycle of differential centrifugation (12,000 g for 10 min followed by 300,000 g for 1 hr). The resulting pellets were suspended in TACM (1.0 ml/6 g of starting tissue). Linear gradients of 10-40% sucrose in TACM were prepared in Beckman SW 41 rotor tubes and used for sucrose density gradient centrifugation (SDGC). One ml preparations from differential centrifugation were layered on each gradient tube, and the gradients were centrifuged for 2.5 hr at 38,000 rpm. The gradients were fractionated from the top into 0.6 ml fractions using an ISCO gradient fractionater. Fractions previously determined (3) to contain infectious TC (fractions 6-9) and BC (fractions 10-12) were concentrated by centrifugation at 250,000 g for 1 hr.

Extraction and electrophoresis of nucleic acids. Nucleic acids were extracted from TC- and BC-containing fractions, representing 10 g of starting tissue in a total volume of 400 µl of 0.04 M Tris-acetate, 0.002 M EDTA, pH 7.5 (TAE) containing 20 µg Proteinase K and 0.5% SDS. Following digestion at 37 C for 45 min, SDS was added to a final concentration of 1.0%, and the mixture was heated to 50 C for 5 min. The preparations were extracted with phenol and chloroform, and the nucleic acids were precipitated with 2.5 volumes of ethanol and 1/10 volumes 2.5 M Na acetate (pH 5.2) at -20 C. Following centrifugation, the pellets were suspended in 10 µl of TAE and analyzed by electrophoresis on 1.0% agarose gels for 2-3 hr at 60 V in TAE and stained with ethidium bromide. Gels were treated with RNAse A (10 µg/ml) in TAE to digest ssRNA and dsRNA or TAE plus 0.3 M NaCl to selectively digest ssRNA.

Electrophoresis of partially purified virus. Fractions from SDGC-containing TC or BC were subjected to electrophoresis at 80 V for 4 hr at 4 C on a 0.5% agarose gel in TAE containing 0.5% 2-ME and staining overnight with ethidium bromide. The virus-containing area of the gel, previously determined to be 0.5 to 1.0 cm from the sample wells, was cut out and electroeluted in dialysis tubing for 4 hr at 75 V using TAE. The eluted virus was concentrated by centrifugation at 250,000 g for 1 hr. Some agarose gels were blotted onto nitrocellulose membranes using TAE as the transfer buffer and procedures described for Southern transfer of DNA.

Serology. An antiserum was produced by COCALICO Biologicals, Inc., P. O. Box 265, Reamstown, Pennsylvania in rabbits, using virus particles purified by electroelution from agarose gels as the inject antigen. Serologically specific electron microscopy (SSEM) of CRSV was as previously described (3). Immunostaining of nitrocellulose membranes was done using the ProtoBlot Western Blot AP System following instructions supplied by the manufacturer (PROMEGA, 2800 S. Fish Hatchery Road, Madison, Wisconsin).

RESULTS

Nucleic acid preparations from BC fractions from CRSV-infected tissue were found to contain both ssRNA and dsRNA (Fig. 1, 2) when assayed by agarose gel electrophoresis and digested with RNAse with or without the addition of NaCl to 0.3 M. Comparable RNAs were not found in corresponding SDGC fractions of healthy (Fig. 1) or citrus tristeza virus (CTV)-infected citrus (Fig. 3).

In preparations that were subjected to SDGC the day after preparation of the original extract BC dsRNA was not seen, whereas BC ssRNA (Fig. 2) was clearly present.

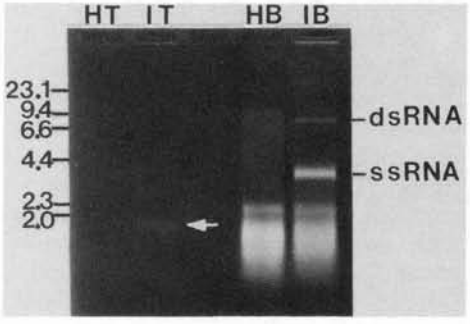

Fig. 1. Agarose gel electrophoresis of nucleic acids from top component (TC) (lanes HT and IT) and bottom (BC) (lanes HB and IB) of healthy and CRSV-infected *C. quinoa*. TC and BC were partially purified by differential and sucrose density gradient centrifugation, all done in one day. TC was further purified by agarose gel electrophoresis. Figs. 1-3 are of nondenaturing 1% agarose gels, and HindIII fragments of lambda DNA were used as size markers.

The molecular weight of BC ssRNA was estimated at between 3.0 and 4.0 x 10^6 when compared with CTV and tobacco mosaic virus RNAs on 1.0% nondenaturing agarose gels (Fig. 3). Comparison with DNA standards indicated a molecular weight of approximately 6 x 10^6 for BC dsRNA.

A single band was observed in a nucleic acid preparation from TC purified by SDGC and agarose gel

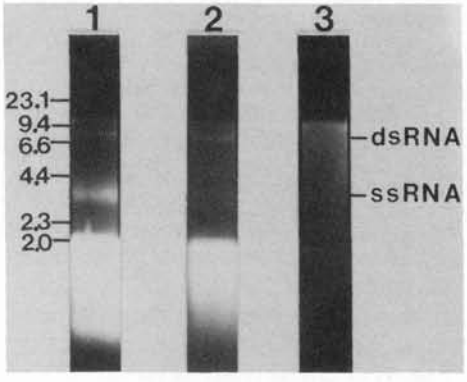

Fig. 2. Agarose gel electrophoresis of nucleic acids of bottom component from a partial purification of CRSV-infected *C. quinoa* done in one day. Lane 1, no RNAse treatment; Lane 2, after treatment with RNAse in 0.3 M NaCl; Lane 3, after treatment with RNAse with no NaCl.

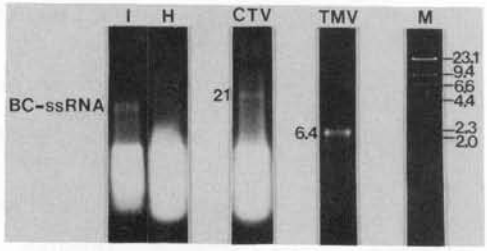

Fig. 3. Agarose gel electrophoresis of nucleic acids of bottom component from partial purifications of CRSV-infected (I) and healthy (H) *C. quinoa* done over two days. ssRNA from citrus tristeza virus (21 kb) and tobacco mosaic virus (6.4 kb) are shown for comparison. Hind-III fragments of lambda DNA are shown in lane M.

electrophoresis (Fig. 1). This band was readily digested by RNAse in low salt (not shown), indicating it was ssRNA. It was not observed in similar preparations from healthy tissue.

Nucleic acid preparations of crude extracts, partially purified preparations, or mixtures of appropriate SDGC fractions in all cases failed to produce a single lesion in infectivity assays. For example, an extract of CRSV-infected tissue in TACM treated with Bentonite (10 mg/g of tissue), SDS to 1%, and phenol and precipitation with ethanol produced no lesions when doubling dilutions were assayed on *C. quinoa*. Corresponding dilutions (1/8 through 1/64) of the untreated extract gave confluent, 78, 13, and 2 lesions/leaf respectively.

The antiserum, produced to CRSV purified by SDGC and agarose gel electrophoresis, readily detected virus particles by SSEM (Fig. 4) and by transfer from agarose gels to nitrocellulose followed by immunostaining (Fig. 5).

DISCUSSION

The filamentous particles associated with CRSV are stable and can be subjected to purification schemes that require several days. Thus, procedures for isolation of CRSV particles of sufficient purity for antisera production and molecular cloning of the virus associated RNAs are now available. Although the particles are stable and

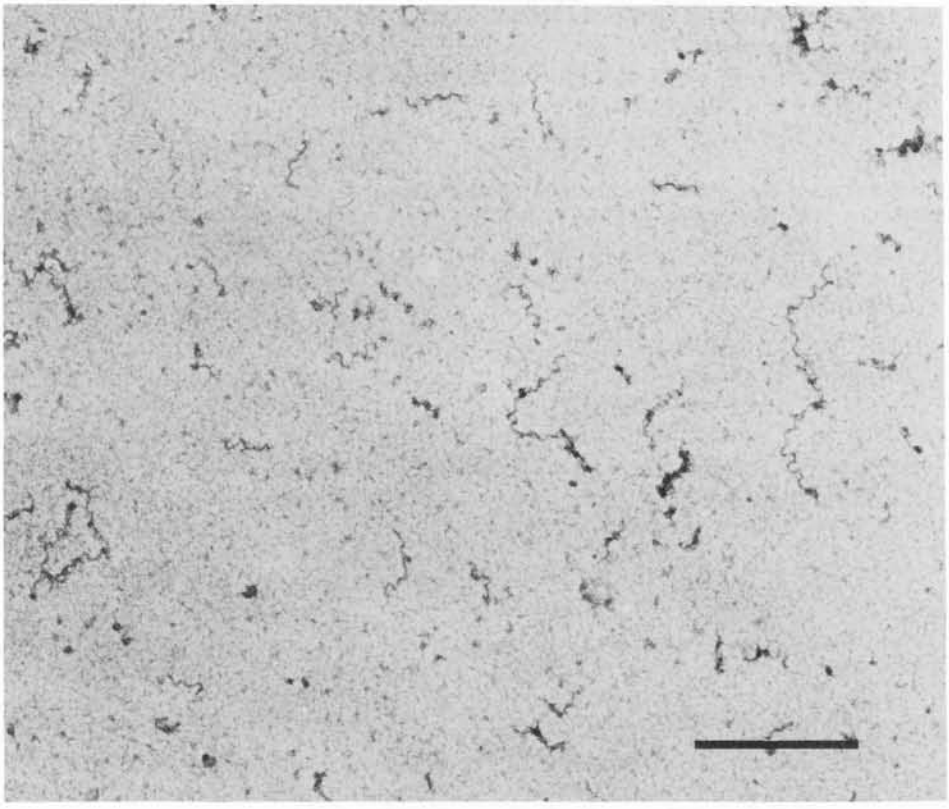

Fig. 4. SSEM of an extract from CRSV-infected *C. quinoa*. The scale bar represents 500 nm.

can be readily isolated, the problem of rapid loss of infectivity in vitro remains. There is a correlation between the loss of infectivity and the failure to detect BC dsRNA after 24 hr at 4 C, which suggests that BC dsRNA is associated with a fragile component that may be required for infectivity.

Serological detection of virus particles by SSEM and by blotting from agarose gels using an antiserum produced to virus purified by SDGC and agarose gel electrophoresis strengthens the case of suggesting that the 48 Kd protein is indeed the capsid protein of CRSV. In addition, this antiserum has been used to specifically detect the 48 Kd protein in western blots or crude extracts of infected tissue (manuscript in preparation).

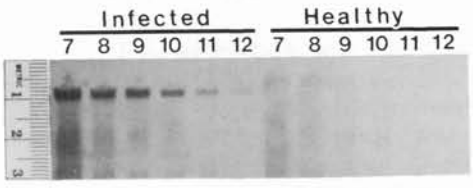

Fig 5. Agarose gel electrophoresis (0.5%) of sucrose density gradient centrifugation fractions 7 through 12 from CRSV-infected and healthy *C. quinoa*. Following electrophoresis the gel was blotted onto nitrocellulose and immunostained.

The following is a summary of the known properties of CRSV:

i) The infectivity associated with the virus is readily separated into top (TC) and bottom (BC) components by SDGC. TC and BC are not infectious alone but are highly infectious when mixed.

ii) Short filamentous particles, a 48 Kd protein and ssRNA are associated with TC.

iii) Long filamentous particles, a 48 Kd protein and ssRNA are associated

with BC. A separate component that contains dsRNA cosediments with BC.

iv) Nucleic acid preparations from the virus are not infectious.

v) Preparations containing filamentous particles, ssRNAs and the 48 Kd protein can be isolated in purification schemes that take several days. Sedimentable BC dsRNA and infectivity diminish rapidly to low levels within 24 hr at 4 C.

vi) It would appear that the filamentous particles contain ssRNA and a 48 Kd capsid protein. The function of the component that contains BC dsRNA and its possible requirement for infectivity are yet to be determined.

vii) Isolates of CRSV and psorosis from Florida, Argentina and Spain were separated into TC and BC that contained a 48 Kd protein. This suggests that ringspot and psorosis are caused by similar or closely related viruses.

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