Cucumo-like Virus Isolated from Cowpea Indicator Plants Manifesting the Citrus Tatter Leaf Virus Syndrome

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ABSTRACT. An icosahedral virus and an elongated virus were found in herbaceous hosts inoculated with citrus plant material carrying the putative citrus tatter leaf virus (CTLV). The elongated virus particle has been always suspected to be the cause of the disease since they were observed by Semancik and Weathers. Several purification attempts to recover this virus from citrus and herbaceous hosts failed precluding any further physico-chemical, biological or serological studies. The icosahedral virus, on the other hand, was more amenable for purificiation in spite of its apparently extremely low concentration in CTLV infected citrus plants. Purification of this virus was never successful when infected citrus was used as the starting material. However, large amounts of icosahedral particles were obtained after transmitting to cowpea and purification carried out using the primary leaves starting to show local lesions 2 or 3 days after mechanical inoculation. The characteristics of the isometric virus allows its tentative classification in the Cucumovirus group according to its physico-chemical properties. Particles were 28 nm in diameter and the electrophoretic mobility of dsRNA extracted from these particles coincides with that of an isolate of CMV. Attempts to transmit the icosahedral virus to citrus plants using mechanical inoculation, slash inoculation and dodder failed.

The putative citrus tatter leaf virus (CTLV) causes a disease that can be economically important in those citrus producing areas where trifoliate orange or its hybrids are used as rootstocks (17). This disease has been always thought to be induced by a virus but efforts by several investigators have failed to identify conclusively the causal agent of the disease (3, 10, 12, 14, 15). Semancik and Weathers (15) found elongated virus particles in symptomatic plants but Koch's postulates were not completed. Similar particles were found by another researcher (14), but he failed to recover sufficient amounts of virus to complete its characterization.

In another attempt to characterize CTLV, Nishio *et al.* (12) purified filamentous flexible particles, 650 × 13 nm, from inoculated *Chenopodium quinoa* plants using molecular permeation chromatography on controlled pore glass beads. They reported the virus as having a single RNA molecule, molecular weight 2.83×10^6 M_r and a coat protein of 27,000 dalton in SDS-PAGE. Since the purified virus immunoreacted with Apple Stem Grooving Virus (ASGV) in an agar double diffusion test and because of its physico-chemical characteristics they suggested that CTLV should be classified as a Capillovirus (7).

We report the purification and characterization of an icosahedral virus from indicator plants showing symptoms corresponding to tatter leaf and citrange stunt. Biological tests are being conducted in order to determine the possible role of this virus in the induction of tatter leaf symptoms on citrus plants.

MATERIALS AND METHODS

The isolates of TLV used in these studies were from a virus collection maintained at the University of California, Riverside. There isolates were collected by different workers at locations in the U.S. and have been perpetuated in Meyer lemon seedlings. The isolates were previously used and their characteristics and history described in detail by Roistacher (13). Bark from the source trees was used to graft-inoculate Etrog citron and Rusk citrange seedlings and grafted combinations of *Citrus excelsa* plants growing on rough lemon rootstock. Leaves from these inoculated plants were used for purification. In a different experiment, purification was performed using cowpea primary leaves

inoculated with selected fractions resulting from purification of CTLV-infected citrus leaves.

Purification protocol. Plant tissue was processed immediately after harvesting it or refrigated before tissue homogenization and all steps were performed at 4C during purification. Extraction buffer containing 0.01 M EDTA, 0.004 M sodium sulfite and 0.1 M sodium citrate, pH 7.2, was added at a ratio of 1:3 to 1:4 depending on the plant tissue used and blended for 3 min in a Waring blender. Chloroform and carbon tetrachloride were added at 125 µl each per ml of suspension and stirred rapidly for 1.5 hr. The low speed supernatant was treated by addition of 6% PEG 8,000 MW followed by a 30-min centrifugation at 13K in a Beckman J2-21 centrifuge using a J14 rotor and 250 ml polypropylene bottles. The pellet recovered was resuspended in 60 ml of 0.04 M phosphate buffer, pH 8.0, by stirring slowly for 2.5 hr. This suspension was clarified by a 5 min centrifugation 7K RPM in a Beckman J2-21 using a J20 rotor and the resulting pellet discarded. A high speed pellet was obtained after a 1 hr centrifugation at 30K RPM in a Beckman Ti35 rotor and resuspended overnight without stirring in 0.015 M phosphate buffer, pH 8.0. The pellet was broken into pieces, agitated and kept in the refrigerator for 4 hr. If green color was present, 1-butanol was added to this suspension, then agitated a few minutes and centrifuged at 5K RPM for 10 min in a JA20 Beckman rotor. Sucrose density gradient centrifugation was performed in a Beckman SW41 rotor at 23K RPM for 2 hr using 10-40% linear gradient prepared the day before and allowed to diffuse overnight. Fractions of the gradient were collected with an ISCO gradient fractionator and immediately used to inoculate cowpea indicator plants. Individual fractions were also analyzed by SDS-PAGE to detect the presence of viral related proteins. Gels were electrophoresed for 2.75 hr at 30 mA, stained with silver nitrate and photographed using type 665 Polaroid film.

Agarose gel electrophoresis of purified suspensions. Aliquots of purified material, before or after sucrose density gradient centrifugation or fractionation, were analyzed by gel electrophoresis in agarose for the presence of virions. Gels of 1% agarose in TAE buffer were prepared in a Idea Mini Slab. Twenty to 40 µl of semipurified suspensions were applied to each well and run at 50V for 2.5 hr. Virion bands were localized by staining with ethidium bromide. Unstained bands in parallel channels were used for electron microscopy and for inoculation of *C. excelsa*, Rusk citrange and cowpea primary leaves. Citrus plants were inoculated by slash inoculation and cowpea by leaf rubbing with cotton swab and carborundum.

Electron microscopy. Grids were prepared using 10 µl of purified material before and after sucrose density gradient or agarose gel electrophoresis of virions, washed with 20 drops of double distilled water, stained with uranyl acetate for 2 min and the excess blotted with filter paper. Grids were observed in a Hitachi 600 electron microscope.

Double stranded RNA extraction. The method described by Morris and Dodds (11) was followed to extract dsRNA from cowpea plants inoculated with the purified icosahedral virus and showing typical CTLV-induced symptoms. All samples were then run in 6% acrylamide gels, electrophoresed at 110 V for 2.5 hr, stained with ethidium bromide and pictures taken with a Polaroid camera loaded with 557 film type in dark room. Samples of dsRNA from citrus tristeza virus (CTV), tobacco mosaic virus (TMV), turnip yellow mosaic virus (TYMV) and cucumber mosiac virus (CMV) were loaded in parallel channels for reference.

RESULTS

Fractions obtained from a discrete zone after sucrose gradient centrifugation from extracts of different citrus sources were infective when assayed

on cowpea primary leaves and produced either local lesions on inoculated leaves or systemic necrosis in stem, petioles and veins of trifoliolate leaves. Local and systemic symptoms were sometimes seen in the same plant. Infective fractions coincided when purification was carried out using symptomatic leaves of Rusk citrange or *C. excelsa*. With both tissues, the virulence of those fractions was extremely low as indicated by the number of local lesions induced in inoculated leaves. However, a dramatic change in virulence to cowpea was seen when cowpea leaves with local lesions were used as inoculum to infect additional cowpea seedlings with primary leaves not completely expanded. These primary leaves were collected 2 or 3 days after inoculation and used as starting material for purification using basically the same protocol as that described for citrus leaves.

Until now no tatter leaf or citrange stunt symptoms have been seen on citrus indicator plants inoculated with a purified CMV-like virus preparation. Initial attempts to reproduce the disease in indicator citrus plants by mechanical inoculation, slash inoculation and dodder have not yet shown any symptoms. However, this virus induces symptoms in cowpea and *C. quinoa* that resemble the symptoms induced by inoculum from citrus leaves infected with CTLV. These include local lesions on inoculated leaves and systemic necrosis of stem and petioles.

SDS-PAGE analysis of fractions obtained after sucrose density gradients showed no difference in protein band patterns when healthy and diseased citrus tissue was used as starting material for purification. However, a distinct protein band was clearly visible when comparable fractions (See Fig. 1, fractions 14 through 25) of healthy and diseased cowpea primary leaves were run on gels. A direct correlation was found between the intensity of that protein band and the number of local lesions induced in primary leaves of cowpea or the intensity of the viral nucleic acid band detected by ethidium bromide stain in agarose gels for virion analysis.

Virion bands obtained in agarose gel without staining with ethidium bromide were used to inoculate cowpea primary leaves and induced spreading necrosis in inoculated leaves. These leaves were subsequently used to inoculate *Chenopodium quinoa* and *Nicotiana clevelandii*. All *C. quinoa* plants developed local lesions and many of them showed strong systemic

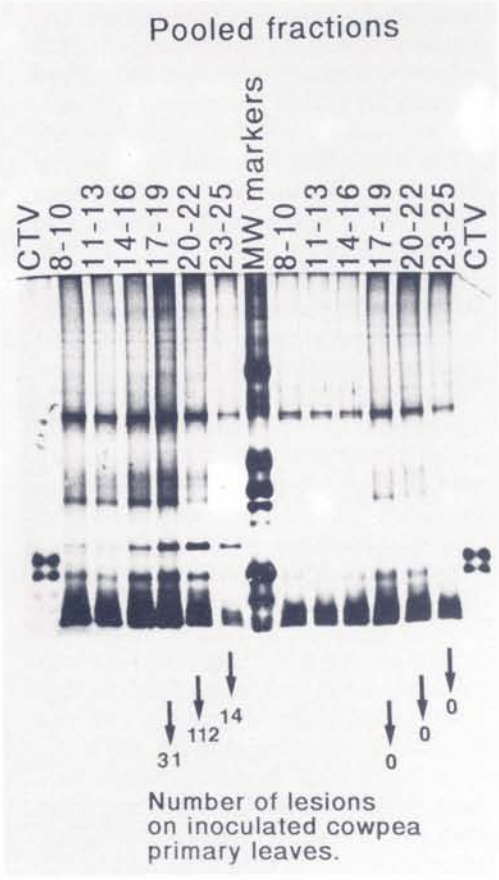

Fig. 1. SDS-PAGE analysis of fractions of sucrose density gradient of partially purified preparations of healthy cowpea and citrus tatter leaf virus-infected cowpea primary leaves 3 days after inoculation. These fractions pooled in groups of three were simultaneously inoculated on cowpea to check infectivity. Lanes 2 to 7 correspond to CTLV-infected cowpea, lanes 9-14 are comparable fractions resulting from purification of healthy cowpea used as controls, lane 8 contains Sigma SDS-7 molecular weights markers and lanes 1 and 15 were loaded with Citrus Tristeza Virus coat protein.

symptoms consisting of twisted stems and distorted leaves in new growth formed after inoculation (Fig. 2). *N. clevelandii* responded with mild symptoms of vein clearing.

It was possible to recover two virus isolates that induce different local lesion symptoms in inoculated primary leaves of cowpea; one that corresponded with the original description of the typical necrotic local lesion (16), which we named type I, and a non-necrotic, chlorotic lesion designated as type II. The type II response displayed an almost circular spot of the same size as the type I lesion that later disappeared or sometimes left tiny necrotic marks. This second lesion type has not been described previously for CTLV. These two isolates induce systemic symptoms in trifoliolate leaves on cowpea seedlings mechanically inoculated on their primary leaves. Both lesion types were isolated by single lesion passages and maintained as true to type isolates in cowpea plants.

Electron microscopy of high speed pellet preparations of both type I and type II sources before sucrose density gradient centrifugation showed the presence of large number of spherical particles of approximately 28 nm in diameter. The same particles were found in specific fractions of the gradient as well as in ethidium bromide-stained bands in agarose gels (Fig. 3).

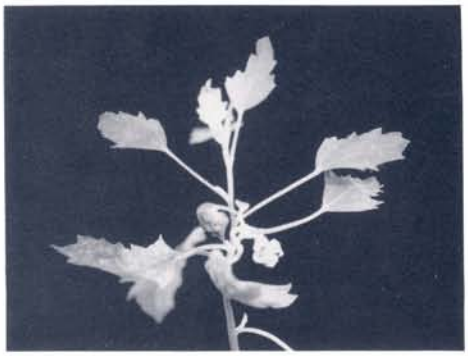

Fig. 2. *Chenopodium quinoa* seedling showing systemic symptoms after inoculation with macerated symptomatic cowpea leaves previously inoculated with icosahedral virus purified by agarose gel electrophoresis.

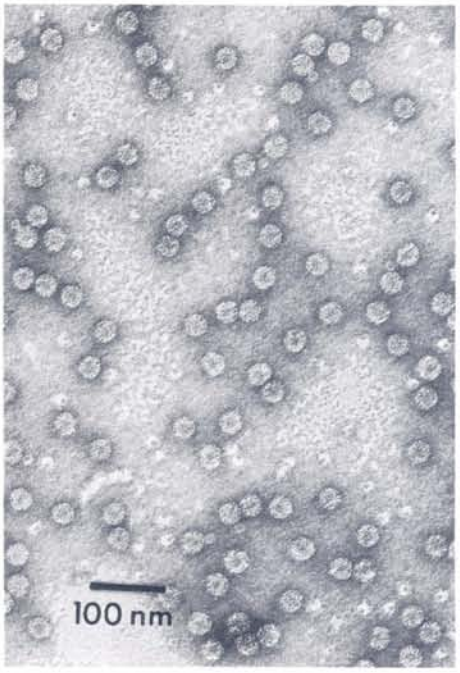

Fig. 3. Electron micrograph of purified icosahedral virions recovered from cowpea primary leaves showing typical citrus tatter leaf virus-induced local lesions.

The dsRNA pattern for the icosahedral virus nearly coincides with that of an isolate of CMV (Fig. 4). Both viruses have four bands with identical electrophoretic mobilities. This strongly suggests that our virus belongs to the cucumovirus group based on the fact that dsRNA patterns like those of CMV are quite unique and, consequently, of great diagnostic value.

DISCUSSION

Even though it is possible to find isolated elongated particles similar to those described by Semancik and Weathers (15), we have not been able to establish the role of those particles in the induction of the "tatter leaf" and "citrange stunt" symptoms. Particles of a similar morphology also have been reported in citrus plants inoculated with psorosis-infected material, but their effect on specific citrus indicators has not been demonstrated.

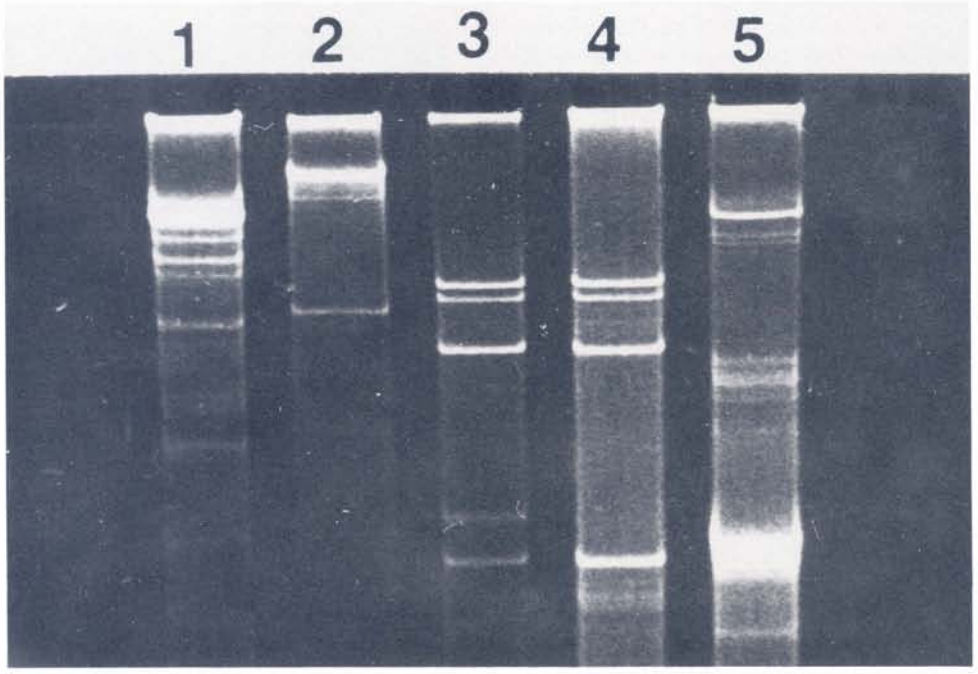

Fig. 4. Double stranded RNA patterns obtained after phenol-SDS extraction and CF-11 cellulose column chromatography of cowpea primary leaves with typical citrus tatter leaf virus-induced symptoms. Gels were made of 6% acrylamide and stained with ethidium bromide. Lane 1 contains turnip yellow mosaic virus, lane 2 citrus tristeza virus, lane 3 cucumber mosaic virus, lane 4 our purified virus and lane 5 tobacco mosaic virus plus satellite tobacco mosaic virus.

Despite the fact that tatter leaf-like symptoms are clearly induced by the isolated icosahedral virus on inoculated cowpea and *C. quinoa* plants this fact does not confirm that the same virus is the causal agent of the disease in citrus plants. Parallel experiments using the elongated particle found by Semancik and Weathers (15) are needed, but this has been impossible to accomplish due to the extremely poor recovery of this type of particle from all infected tissues including citrus and herbaceous alternative hosts.

CMV-like particles have been recovered consistently from cowpea and *C. quinoa* seedlings after inoculation using partially purified preparations from citrus plants showing CTLV symptoms. The virus was recovered in high amounts only after passage of local lesions onto newly developed primary leaves of cowpea and using this material for purification two days after inoculation and before necrosis of the tissues. This resulted in a double benefit of recovering large amounts of virus for characterization as well as a rapid turnover of prime material for purification. Traditionally, virologists have disregarded necrotic local lesion hosts for purification and preferred plants that sustain systemic infection. Even though this concept is generally true, we have demonstrated here that necrotic-local-lesion type hosts can be very advantageous for purification.

It is conceivable that the icosahedral virus causing tatter leaf-like symptoms in herbaceous hosts might be an artifact derived from the use of plants susceptible to CMV. Since CMV is transmitted mechanically, by insect vectors or seedborne, contamination of plants might occur through these avenues as well as by greenhouse accidents. Nevertheless, it also might be possible that a CMV-related virus is actually infecting citrus hosts together with another taxonomically different virus or viruses and its presence is detected only after particle numbers

are increased by passage onto more susceptible hosts. In any case, it is crucial that indicator citrus hosts are included in all experiments with viruses of citrus that involve the use of alternative herbaceous hosts in order to avoid misinterpreting symptoms.

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