Mechanical Transmission of Citrus Tristeza Virus

S. M. Garnsey, D. Gonsalves, and D. E. Purcifull

Horticultural Research Laboratory, U.S. Department of Agriculture, Orlando, FL 32803; University of Florida, Agricultural Research and Education Center, Lake Alfred, FL 33850; and Plant Virus Laboratory, University of Florida, Gainesville, FL 32611, respectively.

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ABSTRACT

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An isolate of citrus tristeza virus (CTV) was mechanically transmitted by a knife-cut inoculation procedure to Etrog citron (*Citrus medica*) receptor plants. The virus was transmitted directly from donor to receptor plants via a contaminated knife blade in 16 of 120 attempts, and from three bark extracts of varying purity to receptor plants in 20 of 67 attempts. Plants infected by mechanical inoculation showed typical systemic symptoms and contained flexuous, threadlike particles (TLP) that are associated with tristeza infection. Control plants remained healthy.

Additional key words: closterovirus, electron microscopy.

Tristeza disease of citrus is caused by an aphid-transmitted, apparently phloem-limited agent generally presumed to be citrus tristeza virus (CTV) (6). The CTV has not been transmitted mechanically (6, 8) or by other means from in vitro sources, and hence many of its basic properties are uncharacterized. Flexuous, threadlike particles (TLP), approximately 2,000 × 10-12 nm are associated with tristeza infection (4, 5) and these have been purified and identified as nucleoproteins (2). The CTV has been included in the closterovirus group (7), although no biological activity has been demonstrated previously for the TLP.

The restricted distribution of CTV in infected hosts has been cited as a probable hindrance to mechanical transmission (5). In our attempts to mechanically transmit CTV, we assumed that the TLP were probably the CTV particles, and that care would be required to physically preserve infectious particles as demonstrated with another closterovirus, the sugar beet yellows virus (9). Inoculation of phloem also was considered as a possible requirement for infection.

In an initial study, a knife-cut inoculation procedure (3) which is highly effective for the direct plant-to-plant transfer of citrus exocortis viroid failed to transmit CTV to 30 Mexican lime [*Citrus aurantifolia* (Christm.) Swingle] and 56 Etrog citron (*C. medica* L.) plants. Later, CTV apparently was transmitted mechanically to two of 123 citron plants inoculated by knife-cut. This occurred in an experiment on citrus exocortis viroid transmission from source plants doubly infected with exocortis and CTV. Other mechanical inoculation procedures, including abrasion of the inner surface of bark flaps and needle puncture, also were tried periodically over several years. Two of 140 citron plants and one of 118 lime plants inoculated with extracts from CTV-infected plants became infected. Infection occurred in plants inoculated on the inner face of Carborundum-dusted bark flaps. Although no infections were observed in control plants, the results were not conclusive.

This paper describes recent experiments in which CTV was mechanically transmitted repeatedly to citrus plants at rates up to 45%.

METHODS AND MATERIALS

The CTV isolate used, coded T-4, was obtained originally from a naturally infected Etrog citron plant. This Florida isolate is free of other known citrus viruses and viruslike diseases. The T-4 isolate causes conspicuous vein-clearing, stunting, and stem-pitting symptoms in Etrog citron and Mexican lime plants, but does not cause seedling yellows (6) in Eureka lemon [*C. limon* (L.) Burm. f.] or sour orange (*C. aurantium* L.).

Bark tissue from 2- to 3-wk-old, new growth of systemically infected citrus plants was used to prepare inocula. Electron microscopy indicated that this tissue contained numerous TLP [50 or more per 75 × 75-μm opening of a 80-μm (200-mesh) grid].

Inoculation was by the knife-cut procedure used for exocortis (3), except that 40 or more cuts per plant were made. Inoculated areas on the stems of receptor plants were wrapped with Stericrepe® (Beacon and Janis, Ltd., London, England) self-adhesive rubber tape (3). The plants were pruned at the time of inoculation and

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periodically thereafter to force new growth.

The receptor plants were rooted cuttings of the OES-4 and Arizona 861 selections of Etrog citron with stems 4-8 mm in diameter.

Inoculated plants were kept in a partially shaded, air-cooled greenhouse with night temperature of 21-24 C, and day temperature of 25-30 C. Pests were carefully controlled.

Evidence for CTV infection in the citron receptor plants was the occurrence of vein-clearing, stem-pitting, and stunting symptoms. Infection was confirmed by examination of negatively stained extracts of young bark or leaf midrib tissue for TLP. Tissue was diced in a solution of 2% potassium phosphotungstate (PTA), pH 6.8, and 0.05% bovine serum albumin. Extracts were incubated on grids coated with carbon-stabilized formvar for approximately 3 min, the excess liquid was removed, and the grids were washed one with stain before examination in a Model 201 Philips® (Philips Electronic Instruments, Inc., Mt. Vernon, NY 10550) electron microscope. Similar procedures were employed to examine inocula. Partially purified extracts were mixed 1:1 (v/v) with stain.

RESULTS

The experiments described were conducted during the spring of 1976, and are summarized in Table 1. Direct transmission of CTV from five donor citrus species or cultivars to citron receptors was achieved (Table 1, treatments 1-6). The knife blade was freshly contaminated by cutting the stem of the donor plant before making each of the 40-60 inoculation cuts.

Because abundant, apparently unbroken TLP were often observed in negatively stained extracts of diced bark, we tested inoculum prepared by a similar extraction procedure. Bark tissue was finely diced in several drops of 0.1M Na_2SO_3 (titrated to pH 8.1 with KH_2PO_4) on a glass slide or glass petri dish placed on crushed ice. The extract was removed by pipette and placed dropwise on the knife blade used to make inoculation cuts. One drop was sufficient to keep the blade wet through a series of 10 cuts. Approximately 60 cuts were made per plant. Six of 20 plants inoculated with this inoculum became infected (Table 1, treatment 7). However, the possibility remained that in the above tests, we had transmitted CTV via bits of tissue or cell organelles and still did not have true mechanical transmission from an in vitro source.

We next tested a partially purified extract from young bark tissue prepared by a method similar to that described by Bar-Joseph et al. (2). Fresh tissue was powdered in the presence of dry ice and ground in 0.1M Tris [tris-(hydroxymethyl) aminomethane] buffer, pH 7.6. The extract was filtered through cheesecloth and partially clarified by low-speed centrifugation. The virus fraction was precipitated from the supernatant liquid by addition of polyethylene glycol (PEG) 6000 and NaCl (2). The precipitate was collected by centrifugation, resuspended, and subjected to a second cycle of PEG precipitation, and the pellet was resuspended in 0.015M potassium phosphate, pH 8.0. The TLP in this preparation were much more numerous than in tissue extracts, but less uniform in length. Drops of the preparation were applied to knife blades and inoculated to receptor plants as in the previous treatment. The inoculum was prepared and tested the same day. Typical CTV symptoms (Fig. 1-B) appeared in nine of 20 plants inoculated (Table 1, treatment 8). Negatively stained extracts from these plants contained typical TLP (Fig. 1-C).

A portion of the partially purified preparation described above also was further purified by centrifugation in a Cs_2SO_4 gradient. The gradient tube was prepared by mixing 9.4 ml of the partially purified prep with 4 ml of 52% (wt/wt) Cs_2SO_4 dissolved in 0.05M Tris-HCl, final pH 8.0, and this mixture was layered on 3.6 ml of the same Cs_2SO_4 solution. The tube was centrifuged at 23,000 rpm at 6 C for 17 hr in a Spinco® SW 27.1 (Beckman Instruments, Inc., Palo Alto, CA 94304) rotor. The gradient was scanned and fractionated with a Model 640 ISCO® (Instrumentation Specialties

TABLE 1. Transmission of citrus tristeza virus (CTV) by knife-cut inoculation to Etrog citron (*Citrus medica*) receptor plants

Treatment	Inoculum preparation[a]	Donor host	Results[b]
1	Intact stem	*C. limon* 'Eureka'	4/20
2	Intact stem	*C. limon* 'Eureka'	3/20
3	Intact stem	*C. excelsa*	1/20
4	Intact stem	*C. medica* 'Etrog'	2/20
5	Intact stem	*C. sinensis* seedling	5/20
6	Intact stem	*C. sinensis* 'Valencia'	1/20
7	Diced bark extract	*C. medica* 'Etrog'	6/20
8	Partially purified	Mixed[c]	9/20
9	Dialyzed TLP[d] zone from Cs_2SO_4 gradient	Mixed	5/27
10	None (control)	None	0/30

[a]A single isolate of CTV (T-4) was used in all tests. In treatments 1-6, a knife blade was contaminated by cutting the stem of a donor plant prior to making each of 40-60 inoculation cuts. In treatments 7-9, liquid inoculum was placed on the blade. The extract in treatment 7 was prepared in 0.1 M Na_2SO_3. The inoculum for treatment 8 was a bark extract partially purified by two cycles of polyethylene glycol precipitation. The same preparation was centrifuged in a Cs_2SO_4 gradient for treatment 9.

[b]Number of plants infected over number inoculated. Infection determined by symptoms and by the presence of the threadlike particles normally associated with tristeza infection.

[c]Tissue from several cultivars.

[d]Threadlike particles (TLP) are the threadlike nucleoprotein particles associated with CTV infection.

Co., Inc., Lincoln, NE 68505) fractionator, and the distinct zone containing TLP was dialyzed against 0.015M K_2HPO_4, pH 8.0. The dialyzed prep was about one-fourth the volume of the partially purified prep and had an $OD_{260} \cong 0.7$. Electron microscopy of this prep showed that it contained numerous TLP of varying

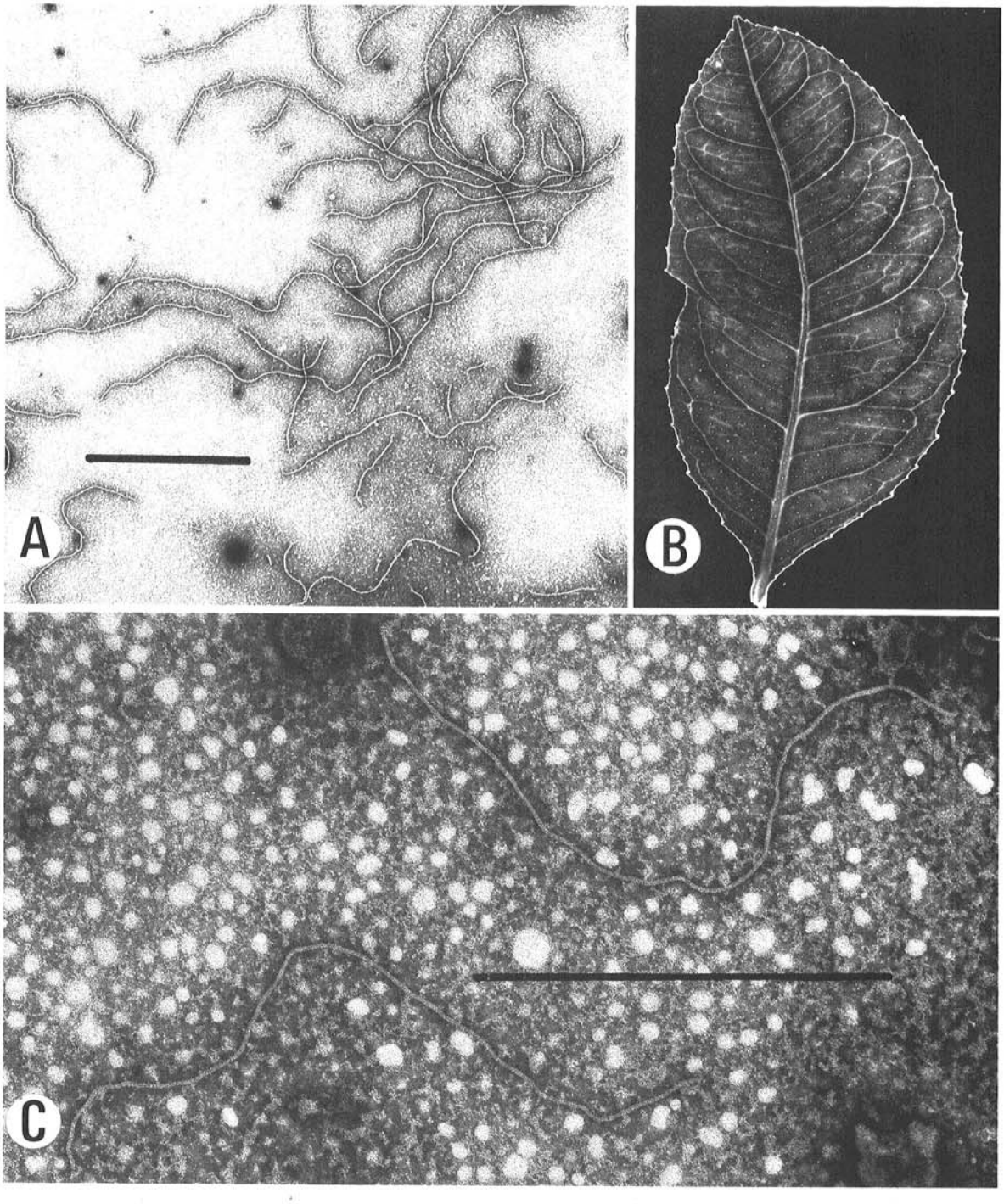

Fig. 1-(A to C). **A)** Electron micrograph of threadlike particles (TLP) of citrus tristeza virus from zone in Cs_2SO_4 gradient dialyzed against buffer and stained with PTA (inoculum for treatment 9, Table 1). The partially purified preparation (treatment 8) had similar, but fewer, particles. Bar = 1 μm. **B)** Systemic vein-clearing symptoms in leaf of an Etrog citron (*Citrus medica* L.) plant mechanically inoculated with partially purified extract (Table 1, treatment 8) from tristeza-infected bark tissue. Symptoms were similar in all plants infected by mechanical inoculation. **C)** TLP in negatively stained extract of bark tissue from a mechanically inoculated citron plant (Table 1, treatment 8). Bar = 1 μm.

lengths and was relatively free of contamination (Fig. 1-A). It also was infectious when assayed as above (Table 1, treatment 9).

The CTV symptoms in all plants infected by mechanical inoculation were typical, and all plants with symptoms contained TLP. Leaf symptoms consistently appeared in the first to third flushes of growth after inoculation (40 to 80 days). Mexican lime plants developed typical symptoms (6) when graft-inoculated with tissue from citron plants infected with CTV via mechanical inoculation.

Noninoculated control plants were selected randomly from the same lot of plants used for the tests above, and were held on the same greenhouse benches. These control plants remained free of symptoms (Table 1), and did not contain TLP. We have observed no natural CTV infections in the thousands of citron and lime stock plants we have grown during the past 10 yr.

DISCUSSION

These results show that CTV can be mechanically transmitted under the proper conditions to citrus receptor plants, and provide substantial new evidence that the TLP are CTV virions.

The ability to mechanically transmit CTV will facilitate studies on relationships among the many strains of CTV described. It will allow additional properties of CTV to be measured and a more complete evaluation of its assignment to the closterovirus group. The molecular weight of the RNA associated with the TLP has been estimated to be approximately 7×10^6 daltons (1). Its properties will be of interest, if found infectious, since it would be considerably larger than sugar beet yellows virus RNA, one of the largest plant virus RNAs reported (1).

Optimum conditions and techniques for mechanical transmission of CTV remain to be identified; however, rapid preparation of inocula which contain numerous nonaggregated and nonbroken TLP and application of these extracts to vascular tissue apparently are desirable. The procedures described here should be a valuable reference point in searches for improved inoculation techniques and for additional hosts of CTV. These results may also encourage further transmission tests with other plant viruses currently not considered to be mechanically transmissible.

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