Purification of Citrus Tristeza Virus from Diseased Citrus Fruits and the Detection of the Virus in Citrus Tissues by Fluorescent Antibody Techniques

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ABSTRACT

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Citrus tristeza virus (CTV) can be purified more easily from diseased fruits than from bark. The purification procedure was: gentle grinding of the pericarp without flavedo of Hassaku or navel orange fruits, clarification with carbon tetrachloride, polyethylene glycol precipitations, and a sucrose density gradient centrifugation. It was shown by electron microscopy that 50% of the particles in the purified preparations were of the normal length (2,000 nm). The ratio of UV absorption at 260 nm and 280 nm ($A_{260/280}$) for purified virus was 1.22. Antiserum against the Hassaku dwarf strain of CTV was prepared in a rabbit given one intravenous and three intramuscular injections of purified preparations. The antiserum reacted to the same dilution end points in complement fixation tests with three strains of CTV: the Hassaku dwarf strain, the seedling yellows strain, and the mild strain. Fluorescent antibodies of the Hassaku dwarf strain of CTV were used for the detection of CTV within tissues of several citrus species. This technique may become a useful tool for the detection of CTV in citrus tissues.

In various species and cultivars of citrus, thread-like particles, approximately 10-12 nm × 2,000 nm, always are associated with the tristeza disease. No similar particles are detected in noninfected plants (8). Bar-Joseph et al. (3, 4) reported the purification of citrus tristeza virus (CTV) from the leaves and bark of citrus trees infected with citrus tristeza disease, by polyethylene glycol (PEG) precipitation and density gradient centrifugation in cesium chloride after fixation with formaldehyde. High concentrations of CTV consistently were found in the albedo of infected Hassaku fruits (11).

In this paper we discuss the purification of CTV from the pericarp (without flavedo) of infected citrus fruits, the preparation of antiserum against CTV, and the detection of CTV in various tissues of citrus trees by means of fluorescent antibody techniques.

MATERIALS AND METHODS

Three strains of CTV, the Hassaku dwarf strain (CTV-HD), the seedling yellows strain (CTV-SY), and the mild strain (CTV-M) were used in these experiments. Ripe fruits from each Hassaku (*Citrus hassaku* Hort. ex Tanaka) tree infected with CTV-HD or CTV-M, and that from the navel orange tree (*Citrus sinensis* Osb.) infected with CTV-SY, were collected from fields in Hiroshima prefecture. These trees also were infected with citrus vein enation virus. Ripe pericarps of healthy Hirokashi No.1 (*C. grandis* Osb. var. *anseikan* Hort. ex Tanaka × *C. tachibana* Tanaka) were used for controls. The flavedo was removed from the pericarp of these fruits, and the pericarps were stored at -70 C until used. The frozen pericarps either were ground in a mortar at -70 C and thawed in five volumes of 0.5 M citrate buffer (pH 6.7) containing 0.3 M sodium chloride and 0.1% thioglycollic acid (grinding method), or homogenized for 3 min in a Model HE universal homogenizer (Nihon Seiki Ltd., Tokyo, 100, Japan) in five volumes of the same buffer (homogenization method) at 4 C. In both cases, the homogenates were centrifuged at 8,000 *g* for 15 min. The supernatant liquid was retained, the pellet was resuspended in four volumes of the same buffer and the suspension was filtered through cheesecloth. Then the filtered juice and the supernatant fluid were mixed. After addition of 6% PEG to the mixture, it was stirred for 1 hr, and centrifuged at 8,000 *g* for 15 min. The pellet was resuspended in 1/10 of the original volume of 0.1 M citrate buffer (pH 7.5). After addition of 10% carbon tetrachloride to the suspension, the suspension-solvent mixture was shaken for 15 min and the emulsion was broken by centrifuging at 8,000 *g* for 15 min. After the addition of 6% PEG and 1.75% sodium chloride to the supernatant fluid, it was stirred for 1 hr and centrifuged at 8,000 *g* for 15 min. The pellet was resuspended in 0.1 M citrate buffer and centrifuged at 8,000 *g* for 10 min. This PEG precipitation procedure was repeated two or three times, and finally the preparation was centrifuged at 120,000 *g* for 60 min. The pellet was resuspended in 0.1 M citrate buffer and clarified by low-speed centrifugation as before. The resulting supernatant fluid was centrifuged at 60,000 *g* for 3 hr in linear density gradient columns of 10-40% sucrose in 0.1 M citrate buffer (pH 7.5). Following centrifugation, the gradient columns were scanned at 254 nm and fractionated with an ISCO density gradient fractionator. The virus-containing zone was collected and centrifuged at 120,000 *g* for 60 min. The virus pellet was

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resuspended in 0.005 M sodium phosphate buffer (pH 7.0). In some cases, further purification was achieved by additional centrifugation in a sucrose density gradient. The purified viruses were stored at −70 C until used.

For electron microscopic examination of the particle length of partially purified virus, preparations were mounted on a carbon-coated collodion grid and negatively stained in neutralized 1% phosphotungstic acid (PTA). Samples for electron microscopy also were prepared by the dip method directly from infected tissues into a drop of 1% PTA on a carbon-coated collodion grid.

Antiserum against CTV-HD was prepared in a rabbit given an intravenous and then three intramuscular injections each of 1 ml of purified virus with an optical density of 1.0 at 260 nm (1-cm light path). Pure γ-globulin from immune serum was conjugated with fluorescein isothiocyanate (FITC) by a dialysis technique (5).

For fluorescence microscopy citrus tissues were fixed in 100% acetone for 1-2 hr at 4 C, and were sectioned (20-30 μm thick) with a razor blade mounted in a Vibratome Model E (Oxford Laboratories, San Mateo, CA 94401). The tissue sections then were stained with fluorescent antibody for 1 hr at 37 C, washed in buffered saline for 30 min, and mounted in buffered glycerol for observation with the fluorescence microscope.

RESULTS

Purification.—Photometric scanning patterns of a preparation of CTV-M after centrifugation in a 10 to 40% sucrose gradient are shown in Fig. 1. Two ultraviolet-absorbing zones were observed. The slower-sedimenting zone also was observed in control preparations from healthy pericarp. The faster sedimenting zone, which was present only in the CTV preparations, contained many threadlike particles without any other type particles when examined by electron microscopy. Centrifugation in rate-zonal density gradients showed that the virus content of a sample prepared by the grinding method was higher than that of a sample prepared by the homogenization method. About half of the purified virus obtained by the grinding method was particles of normal-length (about 2,000 nm) (Fig. 2-A). Virus yields from 150 g of pericarp ranged from 0.5 to 1.2 OD_{260} units/ml. The ratio of UV absorption at 260 nm and 280 nm ($A_{260/280}$) for purified virus was 1.22, suggesting that the nucleic acid content of the particles was approximately 6%.

Serology.—Titer of an antiserum against CTV-HD was 1/1,280, however, it also reacted with preparations from healthy pericarp up to a dilution 1/160 in complement fixation tests. Therefore, the antiserum was absorbed with a healthy preparation. The titer of CTV-HD antiserum after absorption with host components was 1/1,280 with CTV and 1/10 with extracts from healthy pericarp. Purified CTV-HD, CTV-SY, and CTV-M reacted with the antiserum against CTV-HD to equal dilution end points (1/1,280) in complement fixation tests.

Use of fluorescent antibody for the detection of citrus tristeza virus (CTV).—The titer of antibody against CTV-HD conjugated with FITC was 1/640 with CTV and 1/20 with healthy preparations in complement fixation tests. The tissue sections were stained with a 1:60 dilution of fluorescent antibody. The controls used to establish specificity were: (i) healthy citrus tissues treated with labeled antibody prepared against CTV; (ii) citrus tissues infected with citrus vein enation virus, citrus satsuma dwarf virus, and citrus exocortis viroid, respectively, all treated with labeled antibody prepared against CTV; (iii) CTV-infected citrus tissues stained with fluorescent antibody against soil-borne wheat mosaic virus; and (iv) blocking of labeled antibody prepared against CTV with

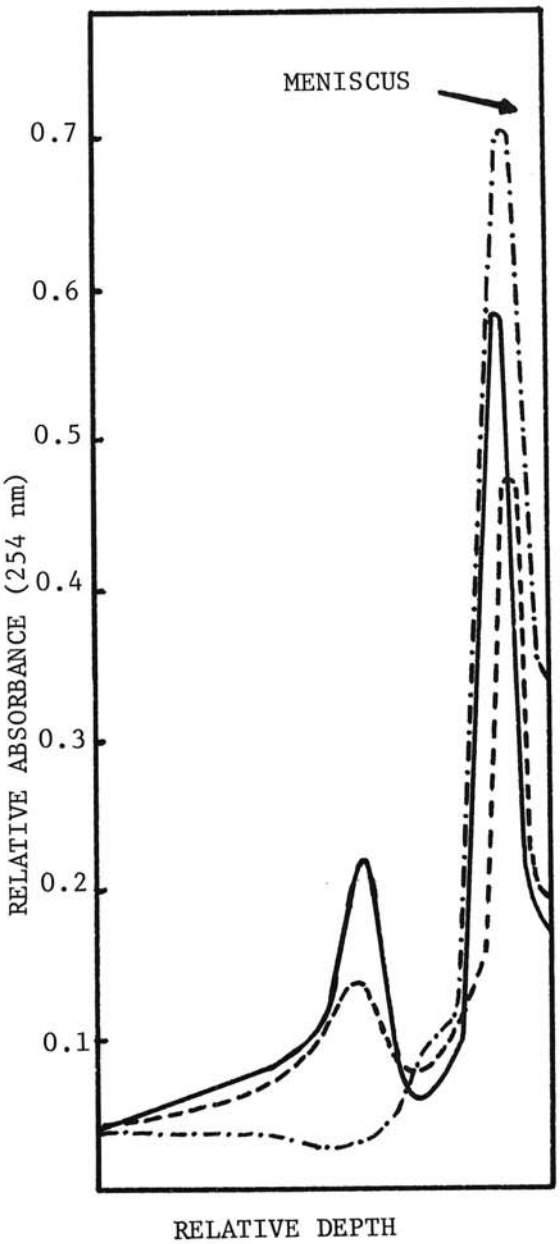

Fig. 1. Photometric scanning patterns of preparations of the mild strain of citrus tristeza virus from 75 g of *Citrus hassaku* paricarps by the grinding method (——) and the homogenization method (------), and of a healthy preparation made from 75 g of Hirokashi No. 1 pericarps (_._._._.) after sedimentation in 10 to 40% sucrose density gradients for 3 hr at 60,000 *g*.

unlabeled antibody to CTV. No fluorescent staining was observed in any of the four controls, whereas CTV stained with homologous fluorescent antibody exibited a characteristic green fluorescence under ultraviolet illumination.

Many brightly fluorescing phloem cells were observed in the vascular bundles of diseased fruits (Fig. 2-B,C). The fruits of a number of *Citrus* spp. [*Citrus unshiu* Marc., *C. reticulata* Blanco, *C. sinensis* Osbeck, *C. iyo* Hort. ex Tanaka, *C. hassaku* Hort. ex Tanaka, an early cultivar of *C. natsudaidai* Hayata, *C. grandis* Osbeck, *C. paradisi* Macf., *C. limon* (L.) Burm., *C. junos* Sieb. ex Tanaka] and a *Fortunella* sp. (kumquat) produced in various areas of Japan, Formosa, and the USA were tested for the presence of CTV by both the fluorescent antibody technique and by electron microscopy. For each sample, vascular bundles from the pericarp of one to three fruits were examined. In every case, there was a complete correlation between the fruits determined by electron microscopy to contain CTV particles and those that gave a positive reaction in the fluorescent antibody test. Of 41 samples examined, 24 from Japan and two from Formosa

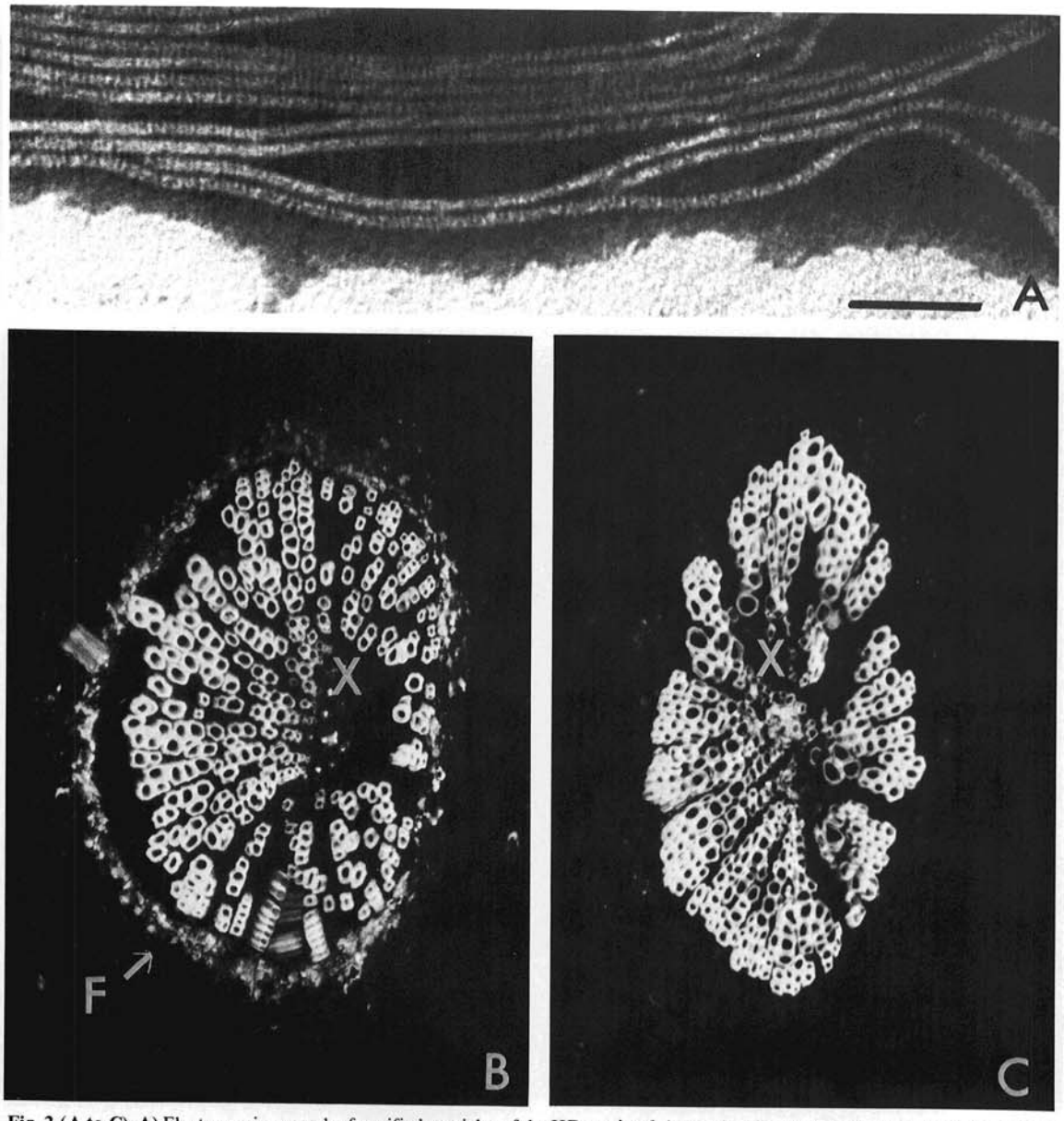

Fig. 2-(A to C). A) Electron micrograph of purified particles of the HD strain of citrus tristeza virus (CTV) after one cycle of sucrose density gradient centrifugation. The scale bar represents 100 nm. (B and C) Fluorescence micrographs of vascular bundles from CTV-infected and healthy citrus fruits: **B)** from *Citrus hassaku* infected with CTV; and **C)** from healthy Hirokashi No.1 (*C. grandis* var. *anseikan* × *C. tachibana*), (F = fluorescence, and X = xylem) (× 300).

were found to contain CTV by both assay methods, and five from Japan and 10 from the United States indexed negative with both techniques. Electron microscopy often required a great deal of time because only a few particles were present on each collodion grid. Therefore, the fluorescent antibody method was more efficient than electron microscopic observation of grids prepared by the dip method for determining whether or not fruits were infected with CTV. Phloem cells in the vascular bundles of stem bark, green twigs, and petioles of various infected *Citrus* spp. always showed characteristic green fluorescence.

DISCUSSION

The purification and concentration of CTV presents several difficulties. The relatively low concentration of particles and their restriction to phloem cells necessitates using large quantities of tissues rich in phloem. Also, CTV has a tendency to aggregate and fragment during purification. The use of stem bark as a source for purification, and a combination of gentle grinding and precipitation by PEG partly overcomes these difficulties (3, 4, 7). However, it is no easy matter to collect large quantities of infected stem bark and to grind it. Pericarp was a better source than the stem bark because it could be obtained in large quantities and could be easily ground. Bar-Joseph et al. (3) reported that if mechanical grinding or homogenization was employed during the extraction procedure of CTV, less than 5% of the particles were in the 1,900- to 2,000-nm range, and more than 60% were shorter than 500 nm. He demonstrated that gentle grinding is essential for the preservation of long particles. We also found that the gentle grinding method resulted in preparation with a relatively high absorbance at 254 nm and a relatively high concentration of normal-length particles. Sensitivity to fragmentation also has been observed with other long filamentous viruses (1, 13).

It has been reported that attempts to achieve additional purification of CTV by sucrose density gradient centrifugation were unsuccessful (4). In the present report, however, concentrated CTV preparations were obtained by sucrose density gradient centrifugation, possibly because of differences in the purification procedure.

Silva et al. (12) prepared an antiserum by using partially purified CTV as antigen. However, differences in the serological reactions of healthy preparations and tristeza preparations were not always evident. There are also more recent reports of the preparation of antisera against CTV and their use for the detection of CTV in citrus (6, 9, 10). However, the fluorescent antibody technique has not been used previously for the detection of CTV.

Our results show conspicuous serological differences between CTV preparations and healthy preparations. Partially purified CTV-HD, CTV-SY, and CTV-M reacted with antiserum against CTV-HD to an equal dilution end point in complement fixation tests. These results indicate that these three strains of CTV have common antigens.

Indexing for CTV is currently done by the lime test, which requires considerable time and greenhouse space. Recently, rapid diagnosis of CTV by electron microscopy of partially purified preparations was reported (2). However, the partial purification required considerable work. The technique using fluorescent antibodies described herein is a very specific, simple, and rapid diagnostic method to detect and identify CTV in infected plants. We believe that this technique can be very useful for detecting diseased plants in the field.

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