

A Simple Purification Method for Citrus Tristeza Virus and Estimation of its Genome Size

M. Bar-Joseph, D. J. Gumpf, J. A. Dodds, A. Rosner, and I. Ginzberg

First and fourth authors, Virus Laboratory, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. Second and third authors, associate professors, Department of Plant Pathology, University of California, Riverside 92521. Fifth author, Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel.

This research was supported in part by grants from the United States-Israel (Binational) Agricultural Research and Development Fund (BARD) 336-80 and the California Citrus Research Board.

We thank C. N. Roistacher, J. Diaz, R. Vasquez, J. Heick, and M. Moscovitz for technical assistance.

Accepted for publication 12 September 1984.

ABSTRACT

Bar-Joseph, M., Gumpf, D. J., Dodds, J. A., Rosner, A., and Ginzberg, I. 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. *Phytopathology* 75:195-198.

The citrus tristeza virus (CTV) purification procedure of Bar-Joseph et al was modified to include a short Cs_2SO_4 -sucrose cushion step gradient that reduced centrifugation time and enabled rapid virus concentration and purification. Electrophoretic mobility in agarose gels of the ssRNA from purified CTV was slower than ssRNA isolated from beet yellows virus, watermelon mosaic virus, and tobacco mosaic virus. The molecular weight of CTV ssRNA was estimated to be $5.4\text{--}6.5 \times 10^6$ daltons. RNA was

extracted from virus particles of various lengths and fractionated by sucrose gradient centrifugation. The RNA was dot spotted onto nitrocellulose paper and hybridized with a plasmid clone containing sequences of CTV cDNA about 600 base pairs long. The hybridization pattern of this probe did not coincide with the CTV antigen distribution as measured by ELISA but showed preferential hybridization with gradient fractions containing normal-size CTV particles.

Additional key words: cesium-sucrose gradient, closterovirus, genomic RNA.

Citrus tristeza virus (CTV) is composed of threadlike particles (TLP) 2,000 nm long and 10–12 nm wide (4,17,19). Garnsey et al (9) transmitted CTV mechanically and associated infectivity with TLP. A molecular weight (MW) of $6.3\text{--}6.9 \times 10^6$ has been predicted for the RNA of CTV (5) from particle length comparisons with two shorter closteroviruses for which normal length RNA MW ratios have been established. Although previous purification procedures (3,4,12,25) provided material suitable for capsid protein characterization and production of antisera (6,10,12,25), they did not provide sufficient intact virus for the characterization of genome RNA.

This paper describes an improved CTV purification procedure that yields intact virus suitable for further biochemical characterization. A brief report of this research has been given (14).

MATERIALS AND METHODS

Viruses. Two CTV isolates obtained from C. N. Roistacher were used for purification. Isolate 512, originally isolated from a naturally infected Meyer lemon at the University of California's South Coast Field Station, causes a mild reaction on lemon (*Citrus limon* (L.) Burm. f.) and strong vein clearing and stem pitting in Mexican lime (*C. aurantifolia* (Christm.) Swingle). Isolate 551, originally isolated from an introduced kumquat [*Fortunella margarita* (Lone.) Swing.], causes a severe seedling yellows reaction in lemon seedlings and strong vein clearing and stem pitting reactions in Mexican limes. Both isolates were increased for purification in etrog citron (*C. medica* L.) seedlings. An additional CTV isolate used only for the hybridization tests was the VT seedling yellows strain (5). Other hosts used for virus propagation were *Claytonia perfoliata* Donn. and *Chenopodium quinoa* Willd. for beet yellows virus (BYV), *Cucurbita pepo* L. for watermelon mosaic virus-2 (WMV-2), and *Nicotiana tabacum* L. for tobacco

mosaic virus (TMV) and cucumber mosaic virus (CMV). The viruses were purified according to published methods for BYV (2), WMV-2 (20), TMV (13), and CMV (18).

Virus extraction and clarification. The improved procedure adapted for the extraction and concentration of CTV particles was modified from the one described by Bar-Joseph et al (3). Bark was peeled from tristeza-infected etrog seedlings when symptoms were pronounced, about 6 mo after graft inoculation, and stored at -20°C until used for virus isolation.

Samples of 40 g bark were ground in a mortar with a pestle in the presence of liquid nitrogen and the frozen powder thawed by placing it in 0.1 M tris (tris hydroxymethyl aminomethane) HCl buffer (pH 7.8) (1 g of bark powder in 5 ml of buffer). The liquid was filtered by expressing through four layers of wet cheesecloth and a layer of paper wipes (Kimwipes). The pulp was reextracted with the same buffer. The two filtrates were combined and centrifuged for 10 min at 4,000 g. The supernatant fluid was collected and again centrifuged for 5 min at 8,000 g to remove debris. Following filtration, the supernatant fluid was collected and again filtered through paper wipes (Kimwipes). Twenty milliliters of a solution of 30% (w/v) polyethylene glycol 6,000 (PEG 6000) dissolved in 0.6 N NaCl plus an additional 2 ml of 20% (w/v) NaCl was added to each 100 ml of the filtrate. After the mixture was allowed to stand for 30–60 min at 4 C, the precipitate was collected by centrifugation at 19,500 g for 15 min. The pellet was resuspended by gentle agitation in 25 ml of 0.04 M sodium phosphate buffer (pH 8.2) (PB) for at least 1 hr and the solution was further clarified by low-speed centrifugation, 5,900 g for 10 min. This suspension was designated as a partially purified viral extract.

Cushion gradient centrifugation. Step gradients were prepared by layering 1 ml each of 0, 15, 22.5, and 30% (w/v) Cs_2SO_4 dissolved in a solution of PB and 10% (w/v) sucrose in 14×87.7 -nm cellulose nitrate tubes. Eight milliliters of partially purified extract was layered on top of the gradients which was then centrifuged for 2.5 hr at 38,500 rpm in a Beckman SW41 rotor at 8 C. Gradient columns were fractionated into 0.6-ml fractions using an ISCO 640 fraction collector and the CTV containing fractions were determined by electron microscopy and enzyme-linked immunosorbent assay (ELISA) (1). Samples for ELISA from each tube were diluted 1/250–1/500 in potassium phosphate buffered

saline (pH 7.4) containing 2% polyvinyl pyrrolidone (PBS-PVP). A drop of each gradient fraction was placed on different carbon-backed grids for 30 sec and the grids were washed with 10 drops of distilled water followed by staining for 15–30 sec with 2% uranyl acetate. Fractions containing the highest concentrations of virus were pooled and mixed with an equal volume of cold PB and clarified by low-speed centrifugation at 8,000 *g* for 10 min. The supernatant fluid was layered on a second cesium-sucrose cushion step gradient, prepared by layering 1.5-ml fractions of 0, 15, 22.5, and 30% (w/v) Cs₂SO₄ dissolved in PB containing 10% (w/v) sucrose. The gradients were centrifuged for 2.5 hr at 38,500 rpm in a Beckman SW41 rotor at 8 C and 0.6-ml gradient fractions were separated as in the first gradient.

RNA extraction and electrophoresis. Virus-rich fractions (~200 ng) of CTV and BYV from the second sucrose-cesium sulphate step gradient were diluted with approximately four volumes of PB, and PEG 6000 was added to a final concentration of 8%. Virus was precipitated by centrifugation (10,000 *g* for 15 min) and the pellet was resuspended in 0.04 tris, 2 mM EDTA (pH 9.0) containing 1% sodium dodecyl sulphate (SDS). Virus preparations could be stored at –60 C as PEG pellets for 1–2 wk before resuspension. Purified WMV-2, TMV, and CMV (5–10 mg/ml) were diluted 1:50 in 0.04 tris, 2 mM EDTA, 1.0% SDS (pH 9.0). All virus suspensions were heated in a 50 C water bath for 15 min before 100 μl aliquots were electrophoresed for 3.5 hr on 0.8, 1.2, and 1.6% agarose gels in 0.04 M tris, 0.02 M sodium acetate, 1 mM EDTA, 0.1% SDS (pH 7.8) at 3 mA/gel. Gels were stained in ethidium bromide (10 ng/ml) for 4 hr, treated with either DNase-I (10 μg/ml; Worthington Biochemicals, New Jersey) and 5 mM MgCl₂ or RNase type 1A (10 μg/ml; Sigma Chemical Co., St. Louis, MO) for 4 hr, restained and then destained. Staining, nuclease treatments, and destaining were all performed in 0.1-strength SSC buffer (0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]).

Sucrose density gradient fractionation. Virus containing fractions from the first Cs₂SO₄ gradients were pooled and dialyzed overnight at 4 C against 0.05 M tris HCl (pH 7.8). The CTV particles were further purified and size fractionated by layering 2 ml of the virus suspension on a 10–40% linear sucrose gradient in 0.05 M tris-HCl (pH 7.8) buffer. After 3 hr of centrifugation at 25,000 rpm in a Beckman SW27 rotor, the gradient was separated into 1.5-ml fractions and the virus concentration in each fraction was estimated by ELISA.

Hybridization of CTV RNA with cloned CTV cDNA. Virus suspensions from each sucrose gradient fraction were diluted 1:5 in

0.05 M tris-HCl (pH 7.8) and the virus particles were pelleted by centrifugation at 45,000 rpm for 2 hr in a Beckman Ti 50 rotor. The viral pellets were resuspended in 100 μl of 50 mM tris (pH 7.5), and 5-μl samples were removed for the HADAS-ELISA (6) assay. RNA was extracted from the purified virus suspensions as described in Rosner et al (21). Aliquots of 3 μl containing RNA prepared from each gradient fraction were dot spotted on nitrocellulose paper presoaked in 20× SSC. A complementary DNA clone to CTV genomic RNA of the VT isolate containing sequences about 600 base pairs long was in-vitro-labeled with ³²P by the nick-translation method (21). Hybridization of the labeled DNA probe with the RNA samples immobilized on the nitrocellulose paper was carried out at 42 C in a buffer containing 50% formamide, 4× SSC, 1× Denhardt solution, and 50 mM Na-phosphate buffer (pH 6.5), essentially as described by Thomas (24).

RESULTS

Purification. After the first cycle of sucrose-cesium sulfate cushion step gradient centrifugation, several light-scattering bands were visible in the gradient tubes. Both observation by electron microscopy and quantification by ELISA showed that the visible opalescent virus band was located immediately below a prominent green band. This pattern of virus distribution was found with the 512 and 551 isolates and also with several other isolates including the VT isolate in Israel. The association between the green band and virus band could be used as a simple marker for locating the virus in similar purifications and gradient centrifugations. The ultraviolet absorption profile obtained by scanning a tube in which the peak fractions from six gradient tubes were recentrifuged on a second sucrose-cesium sulfate cushion gradient are shown in Fig. 1. The CTV particles were concentrated in the fraction corresponding to the peak of absorbance.

Estimation of CTV-RNA molecular weight. The dissociated sample prepared from the sucrose-cesium sulfate density gradient fractions containing CTV was resolved after agarose gel electrophoresis as a broadly stained area of fluorescence (Fig. 2A). Background fluorescence in three other preparations was considerably less than that illustrated. The background fluorescence could be reduced without adversely affecting the CTV-RNA band by DNase treatment of the stained gel (Fig. 2B). Lesser amounts, or none, of this viral RNA was detected in samples prepared from gradient fractions above or below the fraction that contained the most CTV. A DNase treatment of electrophoresed gels improved the resolution of viral RNA of BYV purified by one cycle of sucrose-cesium sulfate density gradient centrifugation (*unpublished*).

The RNAs of CTV (MW = 3.2 × 10⁶), TMV (MW = 2.0 × 10⁶), and CMV (MW = 1.0, 0.7, and 0.3 × 10⁶) were separately resolved by electrophoresis on 1.2% agarose gels (Fig. 2B–D). Only the 1.0 × 10⁶-dalton component of CMV RNA is identified in Fig. 2C–G. The CTV and other viral RNA bands were digested by RNase and fluorescence was lost (*unpublished*). Relative mobilities of CTV-RNA and the standards were compared at three gel concentrations (Fig. 2E–G). The RNA of BYV (MW = 4.2 × 10⁶) was electrophoresed on 1.6% agarose gels (*unpublished*). Molecular weights of the non-denatured CTV-RNA were estimated by extrapolation to be 5.4 × 10⁶ (1.6% agarose), 6.0 × 10⁶ (1.2% agarose), and 6.5 × 10⁶ (0.8% agarose).

Location of CTV in sucrose gradient fractions, detection of virus by ELISA, and hybridization with clone CTV cDNA. CTV virions were size fractionated by sucrose density gradient centrifugation and the virus concentration in each fraction was assayed by ELISA (Fig. 3). The particles were dispersed over a broad band with a distinct peak in fractions 12 and 13. In certain preparations, a second smaller peak was observed in fractions 9 and 10. Electron microscopy of virus particles revealed a variation in size ranging from 100 to 150 nm in fraction 4 to about 2,000 nm in peak fraction 13. RNA was extracted from each fraction of the sucrose gradient, spotted on nitrocellulose sheets, and hybridized with the cDNA clone. This clone was previously shown to hybridize mainly with

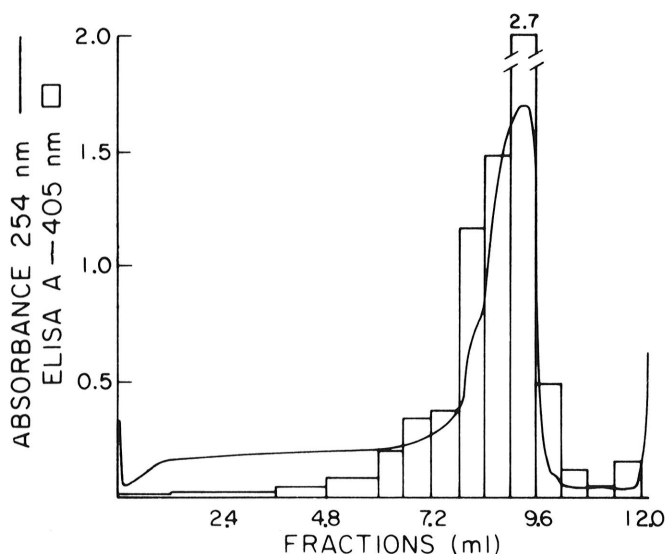


Fig. 1. The distribution of CTV in fractions of the second cesium sulphate sucrose step gradient. The virus-rich fractions from six tubes from the first centrifugation were pooled, diluted with phosphate buffer, and centrifuged for 2.5 hr at 38,500 rpm. Each fraction was diluted 1:500 in PBS-PVP and tested by ELISA (6).

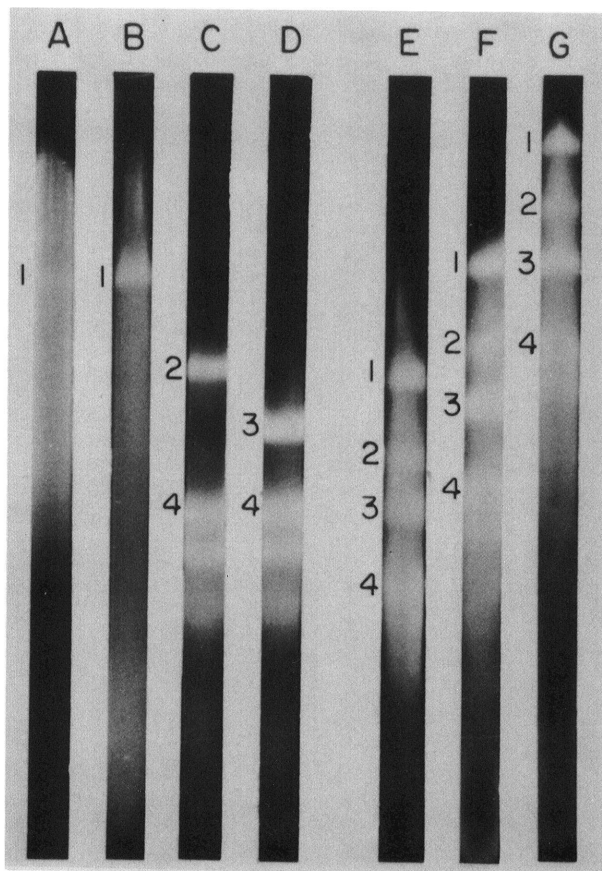


Fig. 2. Relative electrophoretic mobility of RNA from CTV (band 1), WMV (band 2), TMV (band 3), and CMV (band 4) on 0.8% (lane E), 1.2% (lanes A–D and F) and 1.6% (lane G) agarose gels. Resolution of CTV RNA prepared from virus concentrated from density gradient fractions was better in gels treated after electrophoresis with DNase (lanes B and E–G) than in untreated gels (lane A). Electrophoresis was a 3 mA/gel for 3.5 hr. Gels were stained with ethidium bromide.

the full-length virus RNA (21). The hybridization results were quantitated by cutting out the area of each spot and counting the radioactivity bound to the filter. The hybridization pattern of the clone did not coincide with the CTV antigen concentrations as measured by the ELISA test and showed a preferential hybridization with gradient fractions containing predominantly normal-size CTV particles.

DISCUSSION

Following the discovery by Kitajima et al (17) that threadlike particles (TLP) approximately 10–12 nm × 2,000 nm were associated with CTV infections, several groups (3,10–12,19,23,25) attempted to purify these particles. The purification and characterization studies provided substantial information on TLP (3–5,12,23) and established the association of these particles with the citrus tristeza disease (9). The virus particles have been generally purified by several cycles of precipitation with PEG followed by density gradient centrifugation in CsCl (after fixing the virus with formaldehyde) (4) or in a nonfixed form in Cs₂SO₄ (9,12) and in sucrose (11,25). Purification procedures used previously have all been found to be relatively inefficient. For example, serological assays of young bark tissue indicated a CTV titer of up to 100–200 ng/g of tissue (10), but yields of purified virus have been only 0.4–7.5 ng/g tissue (5,11). Low CTV yields may be the consequence of the association of virus with phloem tissues and the tendency of these long particles to aggregate in a nonreversible manner during the precipitation steps. The new purification scheme minimizes the need for repeated CTV pelleting and enables rapid concentration of the virus preparation. These were desirable features because intact particles were required for genome analysis.

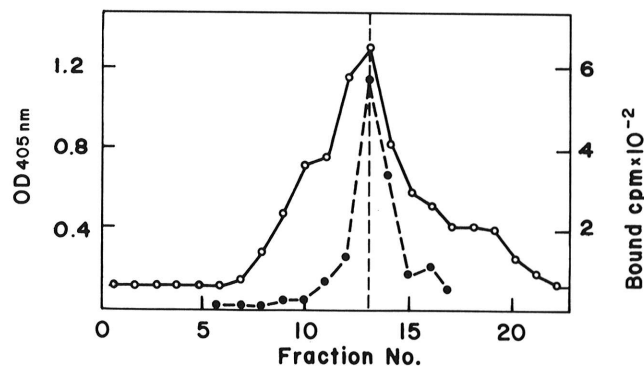


Fig. 3. Hybridization of a ³²P-labeled cDNA probe to RNAs extracted from CTV particles fractionated on a sucrose gradient. CTV was fractionated on a sucrose gradient, and virus in each fraction was quantitated by ELISA (O). RNA was extracted from each fraction and samples (spotted onto nitrocellulose paper) were hybridized with ³²P-labeled clone containing a CTV-cDNA sequence (17). The portions of the nitrocellulose paper containing each spotted fraction were cut out and counts per minute were measured in a liquid scintillation counter (●).

Furthermore, the sucrose-cesium cushion gradient allowed rapid banding of the particles in an easily recognizable gradient fraction.

Closteroviruses have ssRNA genomes of unusually large size for plant viruses. The genome of CTV has not previously been examined by gel electrophoresis. This study has shown that resolution of an RNA molecule of expected size is possible on agarose gels when sufficient amounts of pure virus are available. Attempts to use 2.5% polyacrylamide gels were unsuccessful. An effect of agarose concentration was observed on relative mobility and the highest estimate for the molecular weight of the molecule assumed to be CTV-RNA (6.5×10^6) was obtained from the lowest concentration of agarose (0.8%) suggesting that even this must be considered a minimum value. It is similar to a previous estimate (6.3 – 6.9×10^6) based on particle length (5), and is probably close to the true value. It is in proportion to the molecular weight (13.3×10^6) of the dsRNA RF of CTV determined by electron microscopy (8). This observation, together with the association of the 6.5×10^6 ssRNA with the gradient fraction containing CTV particles, seem sufficient evidence to conclude that the molecule examined is genomic RNA that is derived from normal-length CTV particles. Infectivity has not, however, been demonstrated. This is, if not the largest, among the largest plant viral RNA genomes known. Cloning of CTV cDNA sequences enable us to obtain efficient probes for detection and analysis of minor quantities of CTV RNA (21). The quantitation of the hybridization tests between viral RNA from different gradient fractions with a plasmid clone containing a CTV cDNA sequence about 600 base pairs long showed a preference of hybridization with the lower gradient fractions containing the normal-size CTV particles. It is suggested that the smaller-than-normal population of CTV particles previously considered to consist of randomly fragmented purification artifacts (3) also contain distinct populations of subviral particles not carrying sequences which are found on the cDNA clone. Packaging of subgenomic viral messengers in distinct smaller-than-normal viral particles has been previously reported for TMV. The mRNA of the 30,000-dalton protein of TMV (7) and (in certain strains) also the mRNA of the coat protein (15,16,22) were shown to be separately packaged.

LITERATURE CITED

1. Bar-Joseph, M., Garnsey, S. M., Gonsalves, D., Moscovitz, M., Purcifull, D. E., Clark, M. F., and Loebenstein, G. 1979. The use of enzyme-linked immunosorbent assay for the detection of citrus tristeza virus. *Phytopathology* 69:190-194.
2. Bar-Joseph, M., and Hull, R. 1974. Purification and partial characterization of sugar beet yellows virus. *Virology* 62:552-562.
3. Bar-Joseph, M., Loebenstein, G., and Cohen, J. 1970. Partial purification of virus-like particles associated with the citrus tristeza

- disease. *Phytopathology* 60:75-78.
4. Bar-Joseph, M., Loebenstein, G., and Cohen, J. 1972. Further purification and characterization of particles associated with citrus tristeza disease. *Virology* 50:821-828.
 5. Bar-Joseph, M., Loebenstein, G., and Cohen, J. 1976. Comparison of particle characterization and cytopathology of citrus tristeza virus with other morphologically similar viruses. Pages 39-46 in: *Proc. 7th Conf. Int. Organ. Citrus Virol.* E. C. Calavan, ed. University of California, Riverside, CA. 227 pp.
 6. Bar-Joseph, M., and Malkinson, M. 1980. Hen egg yolk as a source of antiviral antibodies in the enzyme-linked immunosorbent assay (ELISA): a comparison of two plant viruses. *J. Virol. Meth.* 1:179-183.
 7. Beachy, R. N., and Zaitlin, M. 1977. Characterization and in vitro translation of the RNAs from less-than-full-length virus related nucleoprotein rods present in tobacco mosaic virus preparations. *Virology* 81:161-169.
 8. Dodds, J. A., and Bar-Joseph, M. 1983. Double-stranded RNA from plants infected with closteroviruses. *Phytopathology* 73:419-423.
 9. Garnsey, S. M., Gonsalves, D., and Purcifull, D. E. 1977. Mechanical transmission of citrus tristeza virus. *Phytopathology* 67:965-968.
 10. Garnsey, S. M., Gonsalves, D., and Purcifull, D. E. 1979. Rapid diagnosis of citrus tristeza virus infection by SDS immunodiffusion procedures. *Phytopathology* 69:88-95.
 11. Garnsey, S. M., Lee, R. F., and Brlanski, R. H. 1981. Rate zonal gradient centrifugation of citrus tristeza virus. (Abstr.) *Phytopathology* 71:875.
 12. Gonsalves, D., Purcifull, D. E., and Garnsey, S. M. 1978. Purification and serology of citrus tristeza virus. *Phytopathology* 68:553-559.
 13. Gooding, G. C., and Hebert, T. T. 1967. A simple technique for purification of tobacco mosaic virus in large quantities. *Phytopathology* 57:272-276.
 14. Gumpf, J., Bar-Joseph, M., and Dodds, J. A. 1981. Purification of citrus tristeza virus (CTV) on sucrose-cesium sulphate cushion gradients and estimation of its RNA size. (Abstr.) *Phytopathology* 71:878.
 15. Higgins, T. J. V., Goodwin, P. B., and Whitefield, P. R. 1976. Occurrence of short particles in beans infected with the cowpea strain of TMV. II. Evidence that short particles contain the carbon for coat protein. *Virology* 71:486-497.
 16. Hunter, T. R., Hunt, T., Knowland, J., and Zimmern, D. 1976. Messenger RNA for the coat protein of tobacco mosaic virus. *Nature (Lond.)* 260:759-764.
 17. Kitajima, E. W., Silva, D. M., Oliveira, A. R., Muller, G., and Costa, A. S. 1964. Electron microscopical investigations of tristeza. Pages 1-9 in: *Proc. 3rd Conf. Int. Organ. Citrus Virol.* W. C. Price, ed. University of Florida Press, Gainesville. 319 pp.
 18. Lot, H., Marrou, J., Quiot, J. B., and Esvan, Ch. 1972. Contribution à l'étude du virus de la mosaïque du concombre (CMV). I. Méthode de purification rapide du virus. *Ann. Phytopathol.* 4:25-38.
 19. Price, W. C. 1966. Flexuous rods in phloem cells of lime plants infected with citrus tristeza virus. *Virology* 29:285-294.
 20. Purcifull, D. E., and Hiebert, E. 1979. Serological distribution of watermelon mosaic virus isolates. *Phytopathology* 69:112-116.
 21. Rosner, A., Ginzberg, I., and Bar-Joseph, M. 1985. Molecular cloning of complementary DNA sequences of citrus tristeza virus RNA. *J. Gen. Virol.* 64:(In press).
 22. Siegel, A., Hari, V., Montgomery, I., and Kolacz, Z. 1976. Messenger RNA for capsid protein isolated from tobacco mosaic virus-infected tissue. *Virology* 73:363.
 23. Silva, D. M., Oliveira, D. M., and Kitajima, E. W. 1965. Partial purification of tristeza virus. Pages 10-13 in: *Proc. 3rd Conf. Int. Organ. Citrus Virol.* W. C. Price, ed. University of Florida Press, Gainesville. 319 pp.
 24. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Nat. Acad. Sci. USA* 77:5201-5205.
 25. Tsuchizaki, T. A., Sasaki, A., and Saito, Y. 1978. Purification of citrus tristeza virus from diseased citrus fruits and the detection of virus in citrus tissues by fluorescent antibody techniques. *Phytopathology* 68:139-142.