Replication of Citrus Tristeza Closterovirus in Citrus Protoplasts

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ABSTRACT

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The study of citrus tristeza closterovirus (CTV) has been hindered by particle fragility, the inherently low virus titers, and its woody citrus hosts. We report the development of an in vitro *Citrus sinensis* cv. Hamlin protoplast system useful for the study of CTV replication. Full-length CTV genomic RNA from purified virions was inoculated into 'Hamlin' protoplasts in the presence of polyethylene glycol. After 24 h, the protoplasts were assayed for virus replication. Positive- and negative-strand CTV genomic RNA were detected by Northern hybridization using digoxigenin-labeled RNA probes. Three viral-encoded proteins of 20, 25 (capsid protein), and 27 kDa were detected by Western blotting, demonstrating that the viral RNA was translated in vivo. CTV virus particles also were isolated and observed in the electron microscope. This was the first demonstration of the infectivity of CTV RNA and the first report of CTV replication in protoplasts. This system will enable a more detailed study of the replication strategy of this economically important and complex virus.

Additional keywords: electron microscopy, infectious CTV RNA.

Citrus tristeza virus (CTV), a member of the genus *Closterovirus*, is the most economically important virus infecting citrus (1,2,3). Virions are elongate, flexuous rods of 2,000 nm (3). A capsid protein of 25 kDa (3) encapsidates a positive-strand, monopartite RNA of 19,296 nucleotides (nts) (1). CTV exists as many distinct strains, based on a diversity of biological activities in different *Citrus* hosts (4). Plants infected with CTV contain numerous double-stranded RNAs depending on the strain (9), as well as defective RNAs (28). Although the genome organization of one strain is now known (20,30), there is little information available on the replication strategy and the function of the gene products.

The majority of plant viruses infect cells of most tissue types of their susceptible hosts, including mesophyll cells. Closteroviruses, like luteoviruses, are aphid-transmitted and considered to be phloem-limited (2,26). However, studies of CTV inclusion bodies have shown that CTV is not necessarily strictly phloem-limited, since inclusion bodies have been observed occasionally in premeristematic tissue (3,5,7). CTV also has been identified in secondary callus tissue, in which the amount of virus decreases with each transfer of the callus culture (11). Plant viruses that infect most plant cell types, including mesophyll cells, can usually infect mesophyll protoplasts. Protoplasts provide an alternative system to study the infection process and replication strategy of viruses, because they require short incubation times and allow synchronous infections (36). Previous infectivity studies with CTV have been done by slash inoculations of stems (14,23). The genome of CTV, 19,296 nts, is the largest plant virus genome yet characterized. Infectivity has been achieved with preparations containing virions (15,16,23), but not with RNA (1). The complexity of the closterovirus/citrus infection process (i.e., particle fragility, inherently low virus titer, and a host range restricted to woody perennials, primarily citrus and citrus relatives) has hampered infectivity studies.

Recently a "phloem-limited" luteovirus, barley yellow dwarf virus, was found to replicate to high titer in barley mesophyll protoplasts (35). Also, recent research has indicated that direct DNA uptake into citrus protoplasts can be mediated by polyethylene glycol (PEG) (17,21,32,34). These results suggest that a citrus protoplast system might be developed as an alternative approach to study CTV replication. Here we report the infection of sweet orange (*Citrus sinensis* (L.) Osbeck cv. Hamlin) protoplasts using CTV RNA and this system's potential for studying CTV replication.

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MATERIALS AND METHODS

Virion and viral-RNA purification. Virions were purified from bark tissue or leaves of Mexican Lime (*Citrus aurantifolia* (Christm.) Swingle) trees inoculated with the T36 strain of CTV as previously described (24). Full-length CTV genomic RNA was extracted as described by Carpenter et al. (6).

Isolation of citrus protoplasts. Citrus protoplasts were isolated from 'Hamlin' nucellus-derived suspension cells as previously described (17,32). Suspension cultures were maintained in $^1/_2$ & $^1/_2$ medium (EME with $^1/_2$ MT Macro and $^1/_2$ BH3 macronutrients) (17,32) and subcultured every 2 weeks by removing half (25 ml) of the suspension cells and transferring them to a new flask containing 25 ml of fresh $^1/_2$ & $^1/_2$ medium (17,30). Suspensions were

shaken continuously at 140 rpm at 28°C. Suspension cells (1 ml) were harvested 5- to 12-days postsubculture and added to 3 ml of BH3 media (17,32) and 1 ml of enzyme solution (0.7 M mannitol, 0.024 M $CaCl_2$, 0.92 mM sodium phosphate, 6.15 mM morpholineethanesulfonic acid, 0.4 g of Onozuka cellulase (Karlan Chemical Co., Torrance, CA), 0.08 g of pectolyase y-23, 0.4 g of macerase [pH 5.6], volume to 40 ml; filter-sterilized) (17). The solution was incubated in sterile, plastic petri plates (15 × 60 mm) and shaken at 50 rpm for 14 to 16 h in the dark at 28°C. The cell/enzyme mixture was filtered through a sterile, 45-micron stainless steel screen into a sterile, 45-ml glass tube. This solution was transferred to a sterile, 15-ml conical glass tube and centrifuged at 50 × *g* for 10 min. The protoplasts were resuspended in 5 ml of CPW medium (17,32) with 25% sucrose, overlaid with 7 ml of CPW medium with 13% mannitol, and centrifuged for 12 min at 50 × *g*. The viable protoplasts were collected from the interphase and transferred to a fresh, sterile, 15-ml conical glass tube. The protoplasts were washed with 10 ml of BH3 medium (17,30) and collected by centrifugation. The pellet was resuspended in 3 to 5 ml of 0.6 M BH3 medium, and the concentration was adjusted to 1 × 10^6 protoplasts/ml using a hemacytometer.

Approximately 0.5 µg of CTV genomic RNA were added to 1 ml of a PEG cell-fusion solution (40% PEG 1,500, 0.32 M $CaCl_2$, 0.03 M glucose [pH 6.0]). The RNA-fusion solution was added to 1 × 10^6 protoplasts per reaction. After 7 min, the protoplast membranes were stabilized in 1 ml of a mixture of elution solutions A and B at a ratio of 9:1 for 15 min (solution A: 0.04 M glucose, 0.006 M $CaCl_2$, and 10% dimethyl sulfoxide [pH 6.0]; solution B: 0.3 M glycine [pH 10.5]). An equal volume of 0.6 M BH3 medium (17,32) was added to the cells and incubated for 5 min. The solution mixture was carefully removed along the edges, and the PEG-treated protoplasts were washed four times in 0.6 M BH3 medium. The final 0.6-M BH3 medium wash was removed, and the protoplasts were incubated in the dark. The protoplasts were then incubated in the dark in the same solution (0.5 ml) at 28°C.

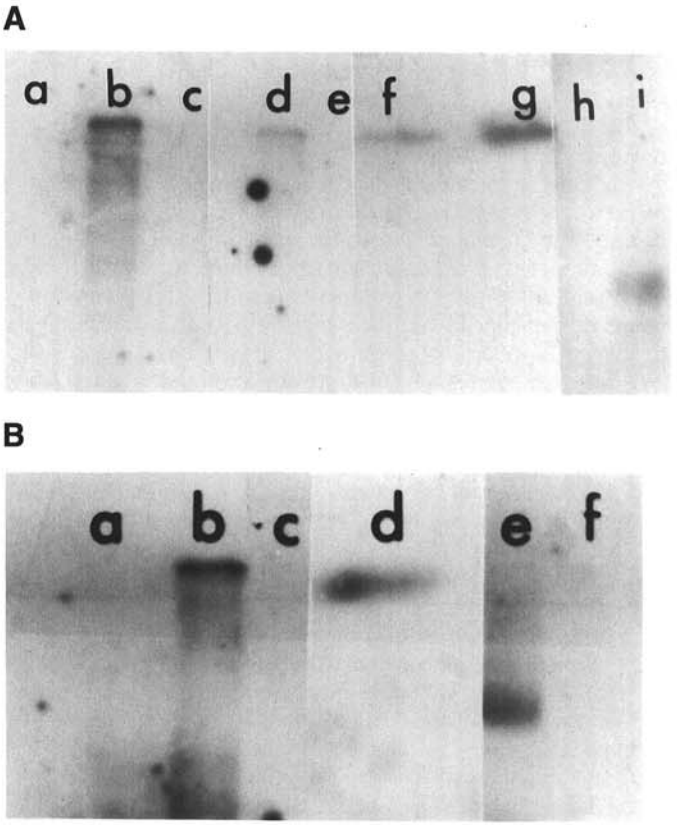

Fig. 1. A, Northern blot for detection of positive-sense strand genomic RNA of citrus tristeza closterovirus (CTV) RNA-inoculated protoplasts. A digoxigenin-labeled riboprobe containing the complementary sequence of CTV capsid protein (672 nucleotides [nts]) was used to detect positive-sense strand CTV genomic RNA. Lane a: Total RNA isolated from mock-inoculated citrus bark tissue. Lane b: Double-stranded RNA isolated from CTV-infected citrus bark tissue. Lane c: RNA isolated from a virion preparation isolated from mock-inoculated citrus bark tissue. Lane d: CTV genomic RNA isolated from virions from infected citrus bark tissue and used as inoculum for protoplasts. Lane e: Double-stranded RNA isolated from mock-inoculated 'Hamlin' protoplasts. Lanes f and g: Double-stranded RNA isolated from CTV RNA-inoculated 'Hamlin' protoplasts. Lane h: CTV cDNA fragments containing the coding sequences of the 25-kDa protein. **B,** Northern blot for detection of negative-sense strand RNA of CTV in CTV RNA-inoculated protoplasts. A digoxigenin-labeled riboprobe consisting of the positive-sense strand gene sequences encoding the 65- and 61-kDa proteins (about 3,500 nts) was used to detect negative-sense strand CTV genomic RNA. Lane a: Double-stranded RNA isolated from mock-inoculated citrus bark tissue. Lane b: Double-stranded RNA isolated from CTV RNA-inoculated citrus bark tissue. Lane c: Double-stranded RNA isolated from mock-inoculated 'Hamlin' protoplasts. Lane d: Double-stranded RNA isolated from CTV RNA-inoculated 'Hamlin' protoplasts. Lane e: CTV DNA fragments containing the coding sequences of the 61- and 65-kDa proteins (6). Lane f: CTV DNA fragments containing the coding sequences of the 25-kDa coat protein.

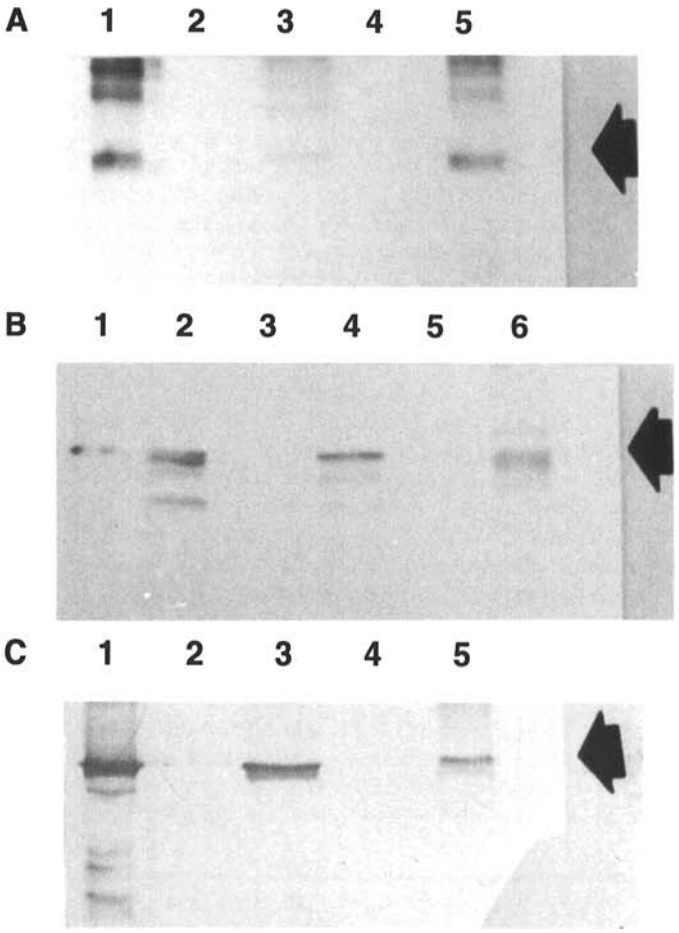

Fig. 2. Detection of virus-encoded proteins in citrus tristeza closterovirus (CTV) RNA-inoculated protoplasts by Western blotting using polyclonal antisera to the **A,** 20-kDa, **B,** 25-kDa, and **C,** 27-kDa CTV proteins. Lane A1: Purified CTV 20-kDa protein expressed in *Escherichia coli*. Lane A2: Mock-inoculated citrus bark tissue. Lane A3: CTV-infected bark tissue. Lane A4: Mock-inoculated 'Hamlin' protoplasts. Lane A5: CTV RNA-inoculated 'Hamlin' protoplasts. Lane B1: Purified CTV coat protein expressed in *E. coli*. Lane B2: Purified CTV T36 virions. Lane B3: Mock-inoculated citrus bark tissue. Lane B4: CTV-infected citrus bark tissue. Lane B5: Mock-inoculated 'Hamlin' protoplasts. Lane B6: CTV RNA-inoculated 'Hamlin' protoplasts. Lane C1: Purified CTV 27-kDa protein expressed in *E. coli*. Lane C2: Mock-inoculated citrus bark tissue. Lane C3: CTV-infected citrus bark tissue. Lane C4: Mock-inoculated 'Hamlin' protoplasts. Lane C5: CTV RNA-inoculated 'Hamlin' protoplasts. Arrows indicate CTV virus proteins detected.

Assays for viral replication. The protoplasts were assayed 24-h postinoculation (PI) for virus replication by Northern blot hybridization, Western blotting, and electron microscopy. As positive controls, viral RNA, viral proteins, and viral particles also were isolated from CTV T36-infected citrus leaf and bark tissue.

To determine if positive- and negative-sense strands of CTV genomic RNA were synthesized in infected protoplasts, Northern blot hybridization experiments were performed using two probes: i) a riboprobe containing the complementary strand sequence of the CTV coat protein gene (672 nts) (25,31) to detect positive-sense strand CTV genomic RNA, and ii) a riboprobe containing the coding sequences (i.e., positive-sense) of the 65 and 61 kDa proteins (about 3,500 nts) to detect negative-sense strand CTV genomic RNA. The probes were labeled using digoxigenin (Boehringer Mannheim Biochemicals, Indianapolis, IN) following the manufacturer's instructions. To detect CTV RNA replication, RNA was purified and used as a control from virions extracted from infected citrus leaves (24) and CTV RNA-inoculated citrus protoplasts at 0- and 24-h PI. Samples were electrophoresed in 0.9% formaldehyde agarose gels (29), the RNAs were then electro-transferred, and the filters were baked at 80°C for 1 h and then analyzed by Northern blot hybridization.

The three CTV-specific open reading frames (ORFs) encoding proteins of 20 (13), 25 (25), and 27 kDa (13) have been cloned and expressed in *Escherichia coli.* These expressed proteins were affinity-purified and used as positive controls. Polyclonal antisera raised against these proteins in rabbits or goats were used in the Western blots of CTV RNA-inoculated protoplasts. Samples for Western blot analysis included total protein extracts from bark tissue of mock-inoculated and CTV-infected Mexican Lime trees 6-months PI and total protein extracts from mock-inoculated and CTV RNA-inoculated 'Hamlin' protoplasts at 0- and 24-h PI. The samples were separated on discontinuous sodium dodecyl sulfate-polyacrylamide gels (22), and the proteins were blotted onto nitrocellulose (33). The blots were blocked in 3% bovine serum albumin overnight at room temperature, incubated in CTV-specific 20 kDa-specific (1:20,000), 25 kDa-specific (1:40,000), or 27 kDa-specific (1:1,000) antisera for 1 h at 37°C, rinsed and incubated in goat anti-rabbit or rabbit anti-goat IgG-alkaline phosphatase secondary antibody conjugate as appropriate (1:20,000) (Sigma Chemical Co., St. Louis) for 1 h at 37°C. Antibody complexes were detected by adding nitroblue tetrazolium and bromochloro-indolyl phosphate (Sigma Chemical Co.).

Since viral RNA was used to inoculate the citrus protoplasts, the production of virus particles also was used to indicate virus replication (27). Using antiserum specific to the CTV coat protein, 'Hamlin' protoplasts were assayed for the presence of virus particles by serologically specific electron microscopy (8) using no. 1053 polyclonal antiserum raised against purified CTV virions (28). Mock-inoculated and CTV RNA-inoculated 'Hamlin' protoplasts isolated at 0- and 24-h PI were centrifuged at 16,000 × *g* for 30 s. The pellets were resuspended in 500 µl of water. As a positive control, virus particles were extracted from bark or leaf tissue of CTV T36-infected Mexican Lime trees (24).

RESULTS AND DISCUSSSION

Probes specific for the positive- and negative-sense strands of CTV RNA hybridized to control double-stranded RNA preparations extracted from CTV-infected citrus bark tissue (Fig. 1A and B, lanes b) and to RNA of the same size as CTV genomic RNA from infected protoplasts (Fig. 1A, lanes f and g; and B, lane d), indicating the in vivo replication of the viral RNA. The levels of synthesis of CTV genomic RNA varied with the viral-RNA preparation used as inoculum as indicated in Figure 1A (lanes f and g). Each probe hybridized to its complement as a positive control (Fig. 1A, lane i; and B, lane e), but not to DNA of a different sequence as a negative control (Fig. 1A, lane h; and B, lane f). Neither probe hybridized to RNA extracted from mock-inoculated protoplasts (Fig. 1A, lane e; and B, lane c) or double-stranded RNA preparations from mock-inoculated citrus bark tissue (Fig. 1A and B, lanes a). An increase in the signal observed between input RNA and at 24-h PI (Fig. 1A, lanes d and f and lanes d and g, respectively) is additional evidence for in vivo replication of CTV, because it is unlikely that the entire amount of inoculum RNA shown in Figure 1A, lane d, was taken up by the protoplasts. Results from immunofluorescence experiments using mock-inoculated and CTV RNA-inoculated 'Hamlin' protoplasts indicated that 1 to 5% of the protoplasts became infected (data not shown). It also should be noted that no subgenomic RNA was detected by Northern hybridization. In this system, subgenomic RNA may be expressed in lower concentrations than CTV genomic RNA and may be below the limits of detection.

Computer-assisted analysis of the nucleotide sequence of the 3′ portion of CTV genomic RNA predicted nine ORFs above 6 kDa (30). To determine whether viral proteins were being translated from CTV RNA in inoculated protoplasts, total protein extracts were prepared from CTV RNA-inoculated protoplasts and analyzed by Western blotting (22,33). Proteins of 20, 25, and 27 kDa expressed in *E. coli* were included in the appropriate blot as positive controls (Fig. 2A, lane 1; B, lane 1; and C, lane 1; respectively). Protein species corresponding to 20 (Fig. 2A), 25 (Fig. 2B), and 27 kDa (Fig. 2C) were detected in CTV RNA-inoculated 'Hamlin' protoplasts (Fig. 2A, lane 5; B, lane 6; and C, lane 5) and bark tissue (Fig. 2A, lane 3; B, lane 4; and C, lane 3), but were not detected in mock-inoculated protoplasts (Fig. 2A, lane 4; B, lane 5; and C, lane 4) and bark tissue (Fig. 2A, lane 2; B, lane 3; and C, lane 2). This indicates that the three viral-encoded proteins were translated from either the input RNA inoculum or newly synthesized CTV RNA-inoculated in the citrus protoplasts. Additional bands of higher-than-expected molecular weight also were occasionally observed in CTV RNA-inoculated citrus proto-

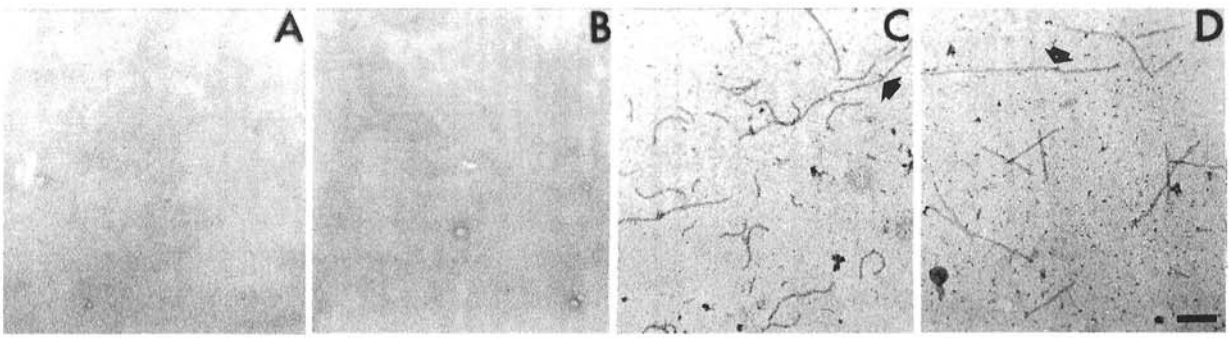

Fig. 3. No antibody-trapped citrus tristeza closterovirus (CTV) particles were detected in **A,** mock-inoculated citrus bark tissue or **B,** mock-inoculated 'Hamlin' protoplasts. Particles were detected in **C,** CTV-infected citrus bark tissue and **D,** CTV RNA-inoculated 'Hamlin' protoplasts. Arrows indicate full-length CTV particles. The bar represents 500 nm.

plasts and bark tissue. During virus infection, virus proteins are often modified. These proteins may represent stable, covalently linked virus proteins complexed to host proteins such as ubiquitin (10,19), to other virus proteins (18), or to readthrough gene products (4).

We also examined the CTV RNA-inoculated citrus protoplasts to determine if mature virus particles were produced. Various lengths of virus particles, including full-length, flexuous rods, were detected from infected bark tissue (Fig. 3C). We also detected rod-shaped virus particles of different lengths from CTV RNA-inoculated protoplasts (Fig. 3D). No particles were detected in mock-inoculated bark tissue (Fig. 3A) or mock-inoculated protoplasts (Fig. 3B). Interestingly, many virus particles detected from CTV RNA-inoculated protoplasts appeared as rigid rods of varying lengths (Fig. 3D), the longer ones comparable to the full-length CTV particles (about 2,000 nm). The possible causes for changes in the morphology of particles from these protoplasts are not known.

In summary, we have developed an in vitro citrus protoplast system that supports the replication of CTV. This was demonstrated by the accumulation of full-length genomic CTV RNA of both polarities, detection of three CTV gene products, and the presence of CTV particles in CTV RNA-inoculated protoplasts. It should be possible now, with this protoplast system, to define the replication strategy and the roles of the many CTV gene products.

LITERATURE CITED

1. Bar-Joseph, M., and Lee, R. F. 1989. Citrus Tristeza Virus. CMI/AAB Descr. Plant Viruses 353.
2. Bar-Joseph, M., Lee, R. F., and Pappu, H. R. 1994. The closteroviruses. Pages 65-85 in: Pathogenesis and Host Specificity in Plant Disease. Histopathological, Biochemical, Genetic and Molecular Bases. Vol. III. Viruses & Viroids. R. P. Singh, U. S. Singh, and D. K. Kohmoto, eds. Elsevier Science Inc., Oxford, United Kingdom.
3. Bar-Joseph, M., Marcus, R., and Lee, R. F. 1989. The continuous challenge of citrus tristeza virus control. Annu. Rev. Phytopathol. 27:291-316.
4. Beier, H., Barciszewska, M., Krupp, G., Mitnacht, R., and Gross, H. J. 1984. UAG readthrough during TMV RNA translation: Isolation and sequence of two tRNAs Tyr with suppressor activity from tobacco plants. EMBO (Eur. Mol. Biol. Organ.) J. 3:351-356.
5. Brlansky, R. H., Lee, R. F., and Garnsey, S. M. 1988. In situ immunofluorescence for the detection of citrus tristeza virus inclusion bodies. Plant Dis. 72:820-826.
6. Carpenter, J. M., Kassanis, B., and White, R. F. 1977. The protein and nucleic acid of beet yellows virus. Virology 77:101-109.
7. Christie, R., and Edwardson, J. 1977. Virus Inclusion Bodies. Fla. Agric. Exp. Stn. Monogr. Ser. 9.
8. Derrick, K. S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56:652-653.
9. Dodds, J. A., and Bar-Joseph, M. 1983. Double-stranded RNA from plants infected with closteroviruses. Phytopathology 73:419-423.
10. Dunnigan, D., Dietzgen, R., Schoelz, J., and Zaitlin, M. 1988. Tobacco mosaic virus particles contain ubiquitinated coat protein subunits. Virology 165:310-312.
11. Duran-Vila, N., Cambra, M., Medina, V., Ortega, C., and Navarro, L. 1989. Growth and morphogenesis of citrus tissue cultures infected with citrus tristeza virus and citrus infectious variegation virus. Phytopathology 79:820-826.
12. Febres, V. J., Pappu, H. R., Anderson, E. J., Pappu, S. S., Lee, R. F., and Niblett, C. L. 1994. The divergent copy of the citrus tristeza virus coat protein is expressed in vivo. Virology 201:178-181.
13. Febres, V. J., Pappu, H. R., Pappu, S. S., Lee, R. F., and Niblett, C. L. 1995. Testing for expression of two citrus tristeza virus genes in infected citrus tissue. (Abstract) Phytopathology 85:1185.
14. Garnsey, S. M., Gonsalves, D., and Purcifull, D. 1977. Mechanical transmission of citrus tristeza virus. Phytopathology 67:965-968.
15. Garnsey, S. M., Lee, R. F., and Brlansky, R. H. 1981. Preparation and stability of infectious citrus tristeza virus (CTV). Phytopathology 71:218.
16. Garnsey, S. M., and Muller, G. W. 1988. Efficiency of mechanical transmission of citrus tristeza virus. Pages 46-54 in: Proc. Conf. Int. Organ. Citrus Virol., 10th. L. Timmer, S. M. Garnsey, and L. Navarro, eds. University of California, Riverside.
17. Grosser, J. W., and Gmitter, J. 1990. Protoplast fusion and citrus improvement. Breed. Rev. 8:339-374.
18. Hayes, R., and Buck, K. 1990. Infectious cucumber mosaic virus RNA transcribed *in vitro* from clones obtained from cDNA amplified using the polymerase chain reaction. Cell 63:363-368.
19. Hazelwood, D., and Zaitlin, M. 1990. Ubiquitinated conjugates are found in preparations of several plant viruses. Virology 177:352-356.
20. Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O., Hilf, M., Koonin, E., Niblett, C., Cline, K., Gumpf, D., Lee, R. F., Garnsey, S. M., and Dawson, W. 1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208:511-520.
21. Kobayashi, S., and Uchimaya, H. 1989. Expression and integration of a foreign gene in orange (*Citrus sinensis osb.*) protoplasts by direct DNA transfer. Jpn. J. Genet. 64:991-997.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.
23. Lee, R. F., Calvert, L. A., and Hubbard, J. D. 1978. Characterization of the coat protein of citrus tristeza virus. Phytopathology 74:801.
24. Lee, R. F., Garnsey, S. M., Brlansky, R. H., and Goheen, A. C. 1987. A purification procedure for enhancement of citrus tristeza virus yields and its application to other phloem-limited viruses. Phytopathology 77:543-549.
25. Manjunath, K. L., Pappu, H. R., Lee, R. F., Niblett, C. L., and Civerolo, E. L. 1993. Studies on the coat protein genes of four isolates of citrus tristeza closterovirus from India: Cloning, sequencing and expression. Pages 20-27 in: Proc. Int. Organ. Citrus Virol., 12th. P. Moreno, J. V. da Graca, and L. W. Timmer, eds. University of California, Riverside.
26. Martin, R., Keese, P., Young, M., Waterhouse, P., and Gerlach, W. 1990. Evolution and molecular biology of luteoviruses. Annu. Rev. Phytopathol. 28:341-363.
27. Matthews, R. 1982. Classification and nomenclature of viruses. Intervirology 17:1-199.
28. Mawassi, N., Gafny, R., Gagliardi, D., and Bar-Joseph, M. 1995. Populations of citrus tristeza virus contain smaller-than-full-length particles which encapsidate subgenomic RNA molecules. J. Gen. Virol. 76:651-659.
29. Miller, K. 1987. Gel electrophoresis of RNA. Focus 9.3:14-15.
30. Pappu, H. R., Karasev, A., Anderson, E. J., Pappu, S., Hilf, M., Febres, V., Eckloff, R., McDaffery, M., Boyko, V., Gowda, S., Dolja, V., Koonin, E., Gumpf, D., Cline, C., Garnsey, S. M., Dawson, W., Lee, R. F., and Niblett, C. 1994. Nucleotide sequence and organization of eight 3′ open reading frames of the citrus tristeza closterovirus genome. Virology 199:35-46.
31. Pappu, H. R., Pappu, S., Niblett, C. L., Lee, R. F., and Civerolo, E. L. 1992. Comparative sequence analysis of the coat protein genes of biologically distinct citrus tristeza closterovirus strains. Virus Genes 7:255-264.
32. Schell, J. L. 1991. Genetic transformation of citrus protoplasts by PEG-mediated direct DNA uptake and the regeneration of putative transgenic plants. M.S. thesis. University of Florida, Gainesville.
33. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76:4350-4354.
34. Vardi, A., Bleichman, S., and Aviv, D. 1990. Genetic transformation of *Citrus* protoplasts and regeneration of transgenic plants. Plant Sci. 69:199-206.
35. Young, M. J., Larkin, P. J., and Miller, W. A. 1989. Infection of *Triticum monococcum* protoplasts with barley yellow dwarf virus. J. Gen. Virol. 70:2245-2251.
36. Zaitlin, M., and Beachy, R. 1974. The use of protoplasts and separated cells in plant virus research. Adv. Virus Res. 19:1-35.