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Amplification of *Citrus Tristeza Virus* from a cDNA Clone and Infection of *Citrus* Trees

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Isolates of the *Closterovirus, Citrus tristeza virus* (CTV), are populations of disparate genotypes and defective RNAs developed during long periods of vegetative propagation of citrus trees. Because it has not been possible to obtain pure cultures of the virus, it is not known what components of the population are primarily responsible for induction of diseases. We previously developed an infectious cDNA clone from which *in vitro*-produced RNA transcripts could infect protoplasts (Satyanarayana *et al.,* 1999, *Proc. Natl. Acad. Sci. USA* 96, 7433–7438). However, neither the RNA transcripts nor virions from transcript-infected protoplasts were competent for infection of citrus trees. Using a green fluorescent protein-marked virus as inoculum, we found that the ~20-kb RNA from virions or transcripts of cDNA infected only a small percentage of protoplasts (~0.01%), but virions could infect more than 80% of the protoplasts. Based on this information, we amplified the virus from the cDNA clone (recombinant virus) by successive passages in protoplasts using virions in crude sap as inoculum. By the third to seventh passages in protoplasts, maximal amounts of recombinant progeny virus were produced, which were used for inoculation of small citrus trees by slashing stems in the presence of virion preparations. A relatively high percentage of plants became infected with the recombinant virus from protoplasts, resulting in the first defined pure culture of CTV in plants. The comparative biology of the pure culture of recombinant CTV with that of the parental population *in planta* demonstrated that the recombinant virus retained through all of the recombinant DNA manipulations the normal functions of replication, movement, and aphid transmissibility, and had a symptom phenotype indistinguishable from that of the parental population. Additionally, fulfilling Koch's postulates of the first pure culture of CTV in plants suggested that the major genotype of the CTV T36 population is the primary determinant of the symptom phenotype. We could distinguish no biological contributions resulting from the minor genotypes and defective RNAs of the parental population.

INTRODUCTION

Citrus tristeza virus (CTV) is a member of the genus *Closterovirus* of the *Closteroviridae*, a family of positive-sense RNA viruses that are transmitted by a range of vectors that include aphids, whiteflies, and mealybugs (Bar-Joseph *et al.,* 1979a; Karasev, 2000). The single-stranded RNA genome of CTV is ~20 kilobases (kb), divided into 12 open reading frames (ORFs) that potentially encode at least 19 proteins (Karasev *et al.,* 1995; Karasev, 2000). ORF 1a encodes a large polyprotein (349 kDa) with two domains homologous to papain-like proteases, plus methyltransferase-like and helicase-like domains. ORF 1b is thought to be expressed by a +1 frameshift to produce the putative RNA-dependent RNA polymerase (RdRp). The ten 3′ ORFs are expressed through 3′ coterminal subgenomic RNAs (Hilf *et al.,* 1995).

CTV causes the most economically important virus diseases of citrus. Depending on the virus isolate, the citrus variety, and the rootstock, two disease syndromes can occur. "Decline" results in death of trees on sour orange rootstocks. "Stem pitting" is an abnormal vascular growth often associated with a lack of vigor and reduced yield of specific varieties, regardless of rootstock. However, some CTV strains or populations cause no obvious symptoms. Different genotypes of CTV differ in sequence much more than expected for strains of a virus, particularly in the 5′ regions, where sequence similarity can be less than 50% (Karasev *et al.,* 1995; Mawassi *et al.,* 1996; Albiach-Martí *et al.,* 2000a). Additionally, most isolates of CTV are populations of disparate genotypes, usually with a predominant "master" sequence, but sometimes with near-equimolar mixes (Ayllón *et al.,* 1999a), plus a myriad of defective RNAs (dRNAs) varying in size, abundance, and number (Mawassi *et al.,* 1995; Yang *et al.,* 1997; Ayllón *et al.,* 1999b).

There has been no reliable method to obtain pure cultures of individual components of CTV populations, since there are no local lesion hosts that allow single-lesion transfers at low dilution. Virus populations can be separated by single-aphid transmission, but often cul-

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tures that appear to be uniform display mixtures of disparate components after transfer through different hosts (Albiach-Martí *et al.,* 2000b; d'Urso *et al.,* 2000). Recently, the 5′-terminal regions of 57 isolates from different parts of the world and from different citrus varieties were examined, and most isolates that cause disease syndromes contain more than one sequence type (probably strain) (Ayllón *et al.,* 2000). Because of the lack of pure cultures, it has not been possible to complete Koch's postulates for individual components. It is not known whether induction of diseases is caused by single or multiple components of the populations or whether dRNAs affect disease induction. Answers to these questions are important for development of management strategies. Two strategies being pursued are production of resistant plants based on posttranscriptional gene silencing and mild-strain cross-protection, both of which require targeting sequences of specific components of the population.

Development of methods to obtain pure cultures of CTV genotypes and then to be able to reconstruct populations from known components would allow assignment of disease induction to specific genotypes or combinations. An *in vitro* genetic system would allow the production of "pure" cultures from cDNA clones propagated in bacterial plasmids. Additionally, *in vitro* creation of mutants and hybrids would allow the mapping of disease determinants to specific nucleotide sequences. Using this approach with *Tobacco mosaic virus,* we were able to demonstrate that the coat protein was involved in inducing disease symptoms (Dawson *et al.,* 1988) or host defense mechanisms (Knorr and Dawson, 1988). Since then, this has become an established approach in plant virology.

Faithfully reproducing all of the genes of a ~20-kb RNA virus through recombinant DNA manipulations is a challenge. Such a genetic system has been developed for CTV that allows replication in protoplasts (Satyanarayana *et al.,* 1999). However, it was not known whether the virus from the cDNA clone [recombinant virus (rCTV)] was representative of the parental virus in plants. Genetically analyzing virus–host interactions requires the ability to infect intact plants with *in vitro*-created virus mutants. CTV is representative of a group of viruses with special obstacles that impede production of a manipulatable genetic system for intact plants. CTV has no known herbaceous hosts. The host range is limited to woody plants, *Citrus* spp., and some relatives, in which infection is limited to phloem-associated cells. It is transmitted primarily by aphids and vegetative propagation. Although it is unusual to be able to mechanically inoculate plants with phloem-limited viruses, Garnsey *et al.* (1977) developed methods to inoculate citrus plants with virion preparations on scalpel blades when slashing stems. However, infection required highly infectious virion preparations. Direct inoculation of plants with RNA transcripts of phloem-limited viruses is a more difficult challenge. Previously, plants were successfully inoculated with other phloem-limited viruses by particle bombardment of the cDNA behind an appropriate promoter and by agroinoculation (Leiser *et al.,*1992; Prufer *et al.,*1995). However, bombardment of full-length CTV cDNA has failed so far, and the size (~20 kb) of the CTV genome along with the few usable restriction endonuclease sites in the cDNA greatly increases the difficulty of agroinoculation.

In this study, we report development of a system to inoculate citrus plants with rCTV virions, allowing us to produce the first "pure" culture of CTV in plants and to compare the biological properties of the rCTV to that of the original parental virus population. Full-length RNAs of CTV, from virions or *in vitro*-transcribed RNA from cDNA, infected only a low percentage of protoplasts and produced a minimal amount of progeny virus. However, we found that virions could infect 80% of the protoplasts. We amplified the rCTV progeny virus by successive passage through protoplasts using virions from CTV9-transfected protoplasts as inoculum in subsequent transfers. Progeny virions from highly infected protoplasts were slash inoculated or bombarded into plants with relatively high efficiency of infection. The rCTV retained normal functions of replication, movement, and aphid transmissibility, and had an *in planta* phenotype indistinguishable from that of the parental population.

RESULTS

Failure to obtain infected plants by inoculation with CTV RNA

Because *in vitro* transcripts from the CTV cDNA clone pCTV9 infect *Nicotiana benthamiana* and citrus protoplasts, we attempted to use RNA transcripts directly to inoculate citrus plants by stem-slashing or by bombardment of RNA preparations into bark patches that were then grafted back in place on citrus plants. Previous attempts to slash-inoculate citrus plants with CTV RNA extracted from virions failed, although virions from the same preparations were infectious (Garnsey, unpublished data). Yet, the long flexuous virions are susceptible to breakage and reduction in specific infectivity during purification manipulations. We thought that a higher concentration of full-genomic RNAs produced by *in vitro* transcription might increase the probability of infection. Although the RNA transcripts from pCTV9 infected *N. benthamiana* and citrus protoplasts, all attempts to infect a range of different citrus varieties of different ages using these transcripts failed. In total, approximately 300 plants were inoculated with the total transcript mixture or with transcripts purified by phenol-chloroform extraction followed by ethanol precipitation. None of these plants became infected. A range of other inoculation procedures, including rubbing cambial faces of bark patches

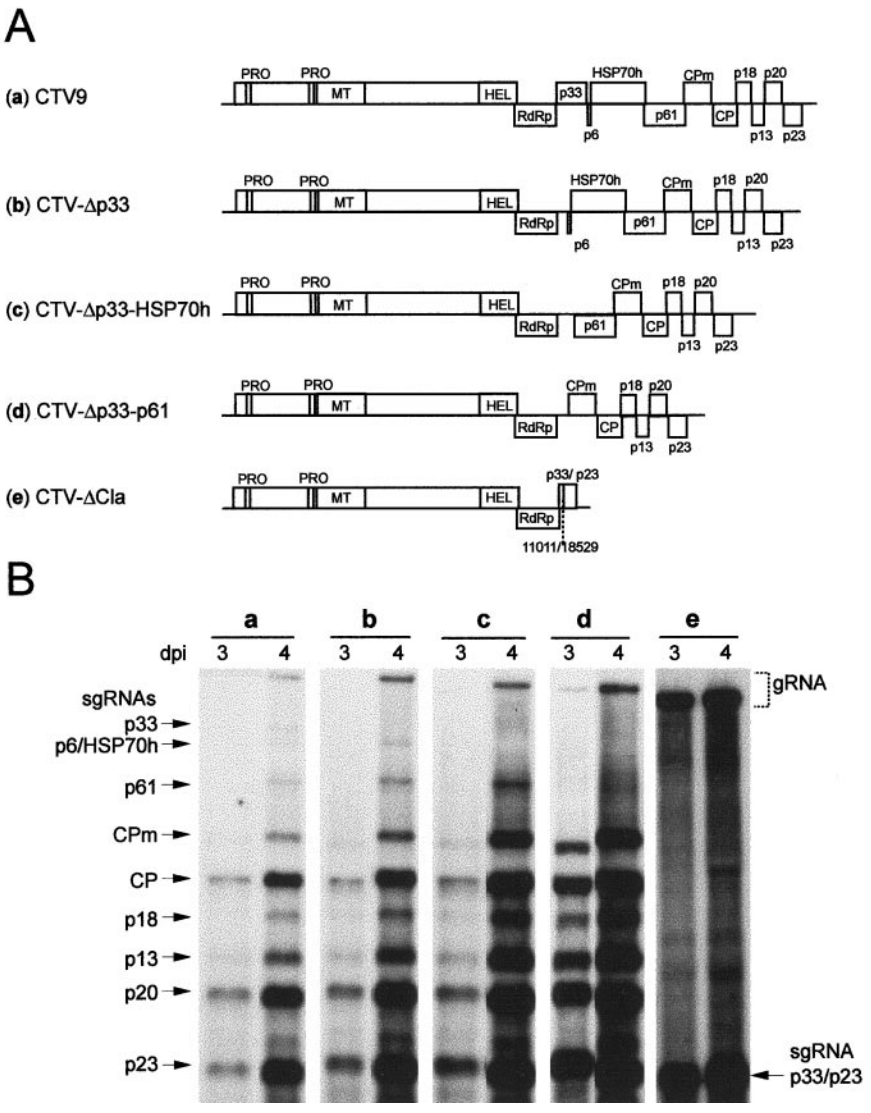

FIG. 1. Effect of CTV RNA size on efficiency of infecting *Nicotiana benthamiana* protoplasts. (A) Genome organization of wild-type CTV (CTV9) (a) and mutants with deletions in 3′ half of the genome (b–e). (B) Replication of CTV9 (a) and deletion mutants (b–e) in *N. benthamiana* protoplasts at 3 and 4 days postinoculation (dpi). Northern blot hybridizations were carried out using a 3′ positive-stranded RNA-specific riboprobe. Position of corresponding subgenomic (sg) RNAs of 3′-end genes are indicated by arrows. sgRNA p33/p23, sgRNA of p33/p23 fusion promoted by p33 sgRNA promoter; gRNA, genomic RNA. Northern hybridization blots of (a–d) were exposed for 1 h and (e) exposed for 20 min.

with RNA transcripts supplemented with different combinations of bentonite, chitosan, carborundum, or glass powders, also failed.

Effect of size of RNA on ability to infect protoplasts

Several observations suggested that the large size of the CTV genomic RNA greatly reduced the ability to infect protoplasts and that perhaps only a small portion of the protoplasts became infected. Smaller RNA transcripts from mutants with large deletions in the ten 3′ genes, which are dispensable for replication in protoplasts, accumulated progeny RNAs to much higher levels than those of full-length or near full-length RNAs. We used CTV deletion mutants (Satyanarayana *et al.,* 1999, 2000) with deletion of p33, p33-HSP70h, p33-p61, and p33-p23 (CTV-ΔCla) genes (Fig. 1A) to examine the ability of different length RNA transcripts (19.3 to 11.8 kb) to infect and replicate in protoplasts. The levels of accumulation of the different mutants were in proportion to the sizes of the deletions (Fig. 1B). The small self-replicating CTV-ΔCla (11.8 kb), with deletion of all ten 3′ genes, accumulated ~50- to 70-fold more than that of full-length CTV9 (19.3 kb). The reduction in full-length RNA accumulation in protoplasts inoculated with RNA transcripts did not appear to be the result of reduced ability of the rCTV to replicate, because protoplasts inoculated with RNA from wild-type virions produced no greater amount of progeny RNAs than did CTV9. These results suggested

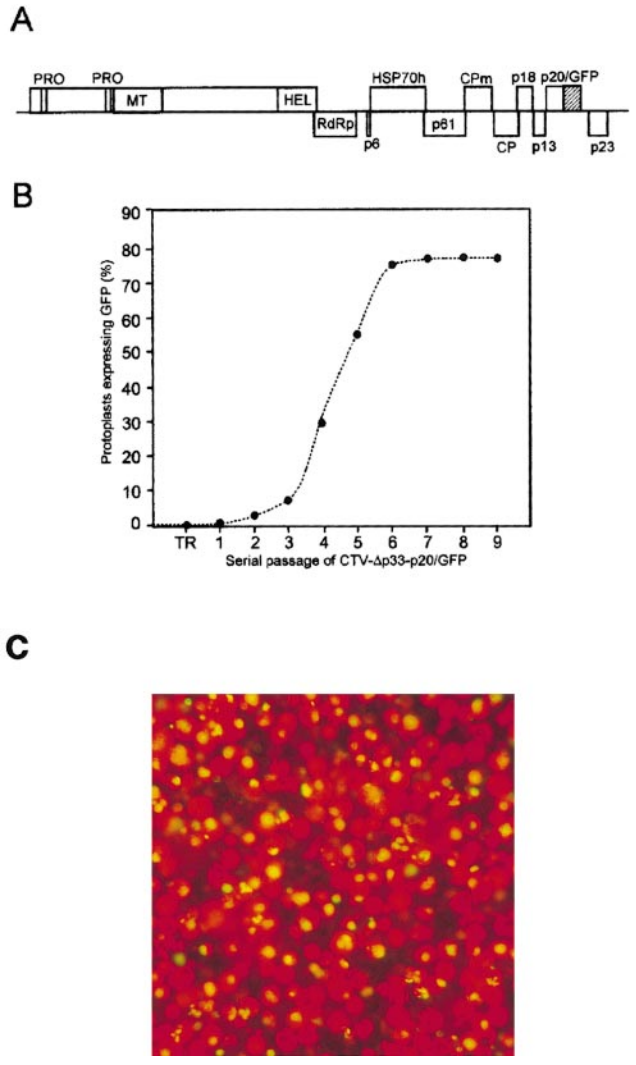

FIG. 2. Expression of GFP from CTV-Δp33-p20/GFP in *N. benthamiana* protoplasts at different serial passages. (A) Schematic diagram of CTV-Δp33-p20/GFP. The GFP ORF was fused in-frame to the 3′-end of p20 ORF in CTV-Δp33 (Gowda *et al.,* 2000; Satyanarayana *et al.,* 2000). (B) Graph showing the percentage of protoplasts infected with CTV-Δp33-p20/GFP, which express GFP at each serial passage in *N. benthamiana* protoplasts. TR, *in vitro* transcripts. (C) A representative field of protoplasts expressing GFP inoculated with virions of CTV-Δp33-p20/GFP at the fifth serial passage (not all of the green fluorescent areas were in focus in any one plane).

that the differences in accumulation of viral RNAs in protoplasts inoculated with different mutants were correlated with differences in the proportions of protoplasts infected, rather than the levels of replication per infected protoplast. To obtain an estimate of the proportion of protoplasts infected, we used a green fluorescent protein (GFP)-tagged virus mutant, which gives a visual signal in infected cells. CTV-Δp33-p20/GFP is a mutant in which the GFP ORF was fused in-frame to the 3′-end of the p20 ORF (Fig. 2A), resulting in a p20-GFP fusion protein that fluoresces brightly when examined under UV light (Gowda *et al.,* 2000). When protoplasts were inoculated with RNA transcripts of CTV-Δp33-p20/GFP, we found only a few fluorescing cells, estimated to be less than 0.01% protoplasts infected.

Infection of protoplasts with crude sap compared to purified virions or RNA transcripts

In initial experiments to infect protoplasts with CTV, we used purified virion preparations or virion RNA as inoculum (Navas-Castillo *et al.,* 1997). Virions were in the range of 1000-fold more infectious than RNA extracted from the purified virions. Subsequently, we realized that the crude (green) sap from bark tissue of infected citrus plants was infectious. In fact, based on the amount of bark tissue used for sap preparation versus virion purification, the crude sap was more infectious per gram fresh weight than the purified-virion preparations. Figure 3A shows a dilution series of crude sap used to inoculate *N. benthamiana* protoplasts. One gram of bark tissue was macerated in 9 ml of 40 mM sodium phosphate buffer, pH 8.2, further diluted with the buffer, and used as inoculum for protoplasts. High levels of replication were detected in protoplasts inoculated with sap diluted up to 1:10,000, an equivalent to 3 μg of infected bark tissue. Comparable replication and accumulation of viral RNAs in protoplasts inoculated with purified virions required an amount equivalent to that obtained from ~600 μg fresh bark tissue (Figs. 3A and 3B). This difference might reflect the poor yield and breakage of the long flexuous virions during purification. However, when applied in sufficient amounts, both sap and purified virions infected protoplasts maximally. Crude sap diluted 1:10,000 (Fig. 3A, 3 μg) provided a level of infection in protoplasts comparable to our maximal infection of RNA transcripts from pCTV9 (Fig. 3C) based on the accumulation of genomic RNAs.

Based on the preceding results, we attempted to inoculate citrus plants with rCTV virions produced in protoplasts, instead of directly inoculating the plants with *in vitro* transcripts of pCTV9. Purified virion preparations or crude sap from protoplasts, which were inoculated with RNA transcripts of pCTV9, were used to inoculate citrus plants by stem-slashing or by particle bombardment. Although the virus replicated in protoplasts based on Northern hybridization analysis (Satyanarayana *et al.,* 1999) and virions were visible by transmission electron microscopy (Satyanarayana *et al.,* 2000), progeny virions from the RNA-transcript-inoculated protoplasts failed to infect citrus plants.

Amplification of rCTV in protoplasts

In an attempt to increase the percentage of infected protoplasts, and thus to increase the amount of progeny virions in a batch of protoplasts, we serially passaged the virions in *N. benthamiana* protoplasts. Crude sap from the protoplasts was used as the inoculum after

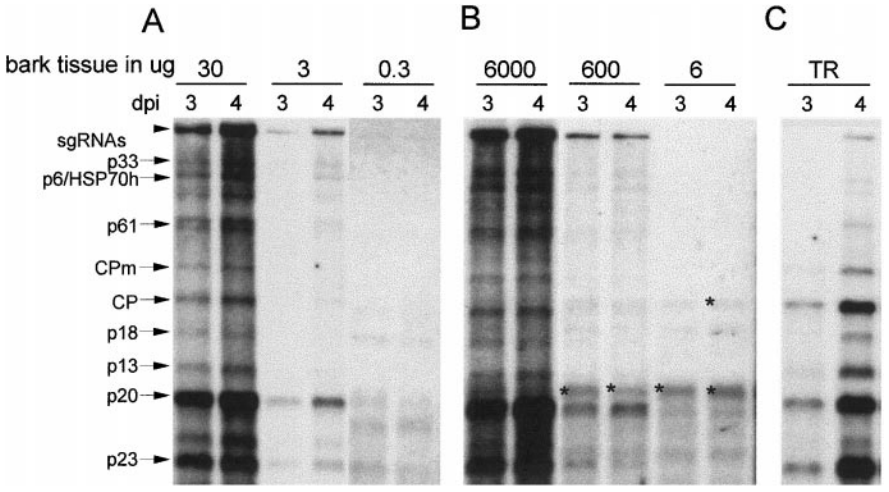

FIG. 3. Comparison of replication of CTV T36 in *N. benthamiana* protoplasts using crude sap extracted from infected bark tissue (A), purified virions from bark tissue from infected plants (B), and *in vitro* transcripts (TR) from pCTV9 (C) as inoculum. The approximate equivalent amount of bark tissue used in 30 μl inoculum is represented in micrograms. The protoplasts were harvested at 3 and 4 dpi and total RNA analyzed by Northern blot hybridization using a 3′ positive-stranded RNA-specific riboprobe. The position of genomic RNA (arrow head) and sgRNAs of corresponding 3′-end open reading frames (arrows) are indicated to the left of the figure. Asterisks indicate the position of nonspecific RNA bands. Northern blots were exposed for 1 h.

infection of the primary batch of protoplasts with RNA transcripts from pCTV9. Figure 4A shows a Northern blot hybridization, demonstrating the relative amounts of viral RNA accumulation in successive passages of protoplasts, each harvested at 4 days postinoculation (dpi). The levels of accumulation continued to increase and reached a maximum after three to seven passages. The maximal level of infection obtained using sap of protoplasts was approximately the same as that from protoplasts inoculated with sap from infected bark tissue. ELISA and Western immunoblot analysis indicated a greater than 160-fold increase in CTV coat protein after three serial passages (Fig. 4B), and EM observations revealed an abundance of virions (Fig. 4C). To obtain an estimate of the proportion of protoplasts infected at each cycle, we passaged the GFP-tagged virus mutant (Fig. 2A) and monitored the proportion of fluorescent cells. Figure 2B shows the percentage of fluorescing protoplasts after each passage and Fig. 2C shows a representative field of protoplasts after the fifth passage with ~60% of protoplasts fluorescing. The percentage of protoplasts fluorescing increased in subsequent passages up to ~80% (Fig. 2B).

Infection of citrus plants by rCTV

The first experiment in which citrus plants were slash-inoculated with sap from the fourth to sixth cycles of protoplasts infected with rCTV from pCTV9 resulted in infection of all three *Citrus* spp. tested; six of eight Mexican limes [*Citrus aurantifolia* (Christm.) Swing.], three of 24 Volkamer lemons [*C. volkameriana* (Pasq.) Tan.], and eight of 18 Etrog citrons (*C. medica* L.) plants became infected. Systemic infection of upper leaves and twigs of Mexican lime plants was detected by ELISA (Bar-Joseph *et al.,* 1979b) as early as 22 dpi and the infected plants exhibited symptoms by 55 dpi. Receptor plants were preindexed by ELISA to eliminate the possibility that any had accidentally become infected during the propagation period. The difference in infection rates probably reflected differences between size and number of slashes between different plants used for inoculation, rather than inherent differences in the sensitivity of these *Citrus* spp. In the next experiment, seven of 11 Etrog citrons and four of five Alemow (*C. macrophylla* Wester) plants were infected by slash-inoculation and the infectivity rate was similar to that for the Mexican lime plants. We subsequently inoculated Etrog citron and Alemow plants by stem-slashing using partially purified rCTV virion preparations, and all five Alemow and four of five Etrog citrons became infected. We also inoculated 11 Etrog citron plants by biolistic inoculation of the cambial face of bark patches with crude sap from the amplified protoplasts infected with rCTV. The inoculated bark patches were grafted back in place in the citrus tree trunk. Four of 11 plants developed systemic symptoms and infection was confirmed by ELISA.

Comparative biology of the rCTV and the parental wild-type population

Mexican lime and Alemow plants initially infected with rCTV from pCTV9 showed characteristic vein clearing and leaf cupping, similar to that induced by the parental wild-type population, CTV T36. Further biocharacterization was done by graft-inoculation of a range of indicator plants: three plants each of Duncan grapefruit (*C. paradisi* Macf.), sour orange (*C. aurantium* L.), Madam vinous

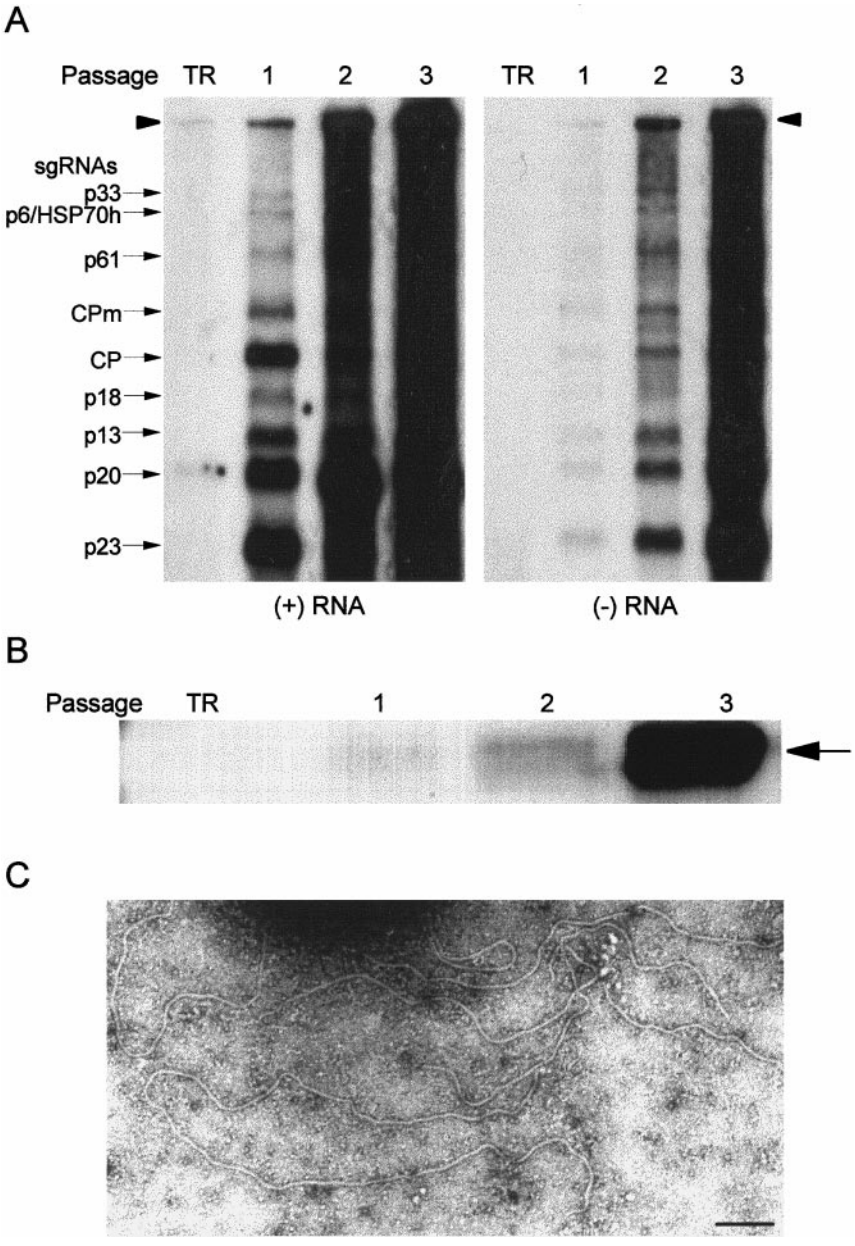

FIG. 4. Amplification of rCTV in *N. benthamiana* protoplasts. (A) Replication of CTV9 from *in vitro* transcripts (TR), and subsequent first, second, or third passage of virions in crude sap from protoplasts at 4 dpi. Northern blot hybridizations were carried out using 3′ positive- and negative-stranded RNA-specific riboprobes. Position of genomic RNA is indicated by an arrowhead and corresponding sgRNAs of 3′-end genes are indicated by arrows. Note the several-fold increase in accumulation of genomic RNA and sgRNAs from passage to passage using crude sap containing rCTV virions as inoculum. (B) Western immunoblot analysis of CTV CP from protoplasts transfected with TR or subsequent first, second, and third passage of rCTV virions at 4 dpi. The position of CP is indicated by an arrow. (C) Negative-stained electron micrograph showing abundance of virus particles from second passage of rCTV virions. Bar, 100 nm.

sweet orange [*C. sinensis* (L.) Osbeck.] as seedlings, plus three each of a clonal propagation of Mexican lime on Alemow rootstock and Valencia orange [*C. sinensis* (L.) Osbeck.] grafted to sour orange rootstocks. All sets of plants were graft-inoculated with rCTV or the parental wild-type CTV T36 population. Symptoms of rCTV- and wild-type T36-infected plants were compared to each other and to healthy control plants. In addition to typical strong vein clearing and leaf cupping symptoms (Timmer *et al.,* 2000) both rCTV and the parental population produced stem pits in the twigs of Mexican lime plants 6–9 months after inoculation. Both infected sets of sour orange and the Duncan grapefruit seedlings exhibited a moderate "seedling yellows" response and the Valencia/sour orange combination plants were stunted and chlorotic at 6 months after graft inoculation. No symptoms were seen on sweet orange seedlings, and the Duncan grapefruit did not produce stem-pitting symptoms similar

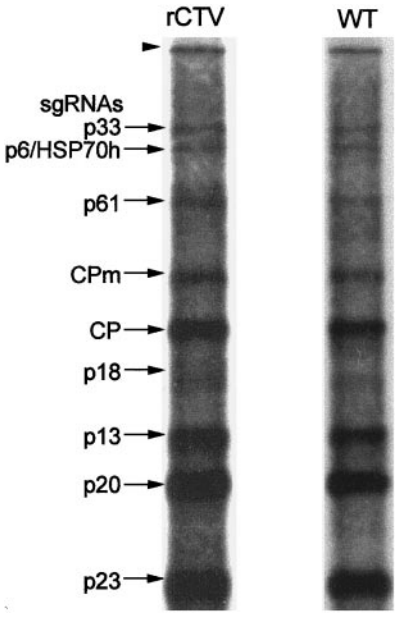

FIG. 5. Northern blot hybridization analysis of dsRNA preparations extracted from rCTV and wild-type CTV T36 isolate (WT)-infected Mexican lime [*Citrus aurantifolia* (Christm.) Swing.] plants. Northern blots were probed with 3′ positive-stranded RNA-specific probe.

to the wild-type CTV T36 isolate. Thus, the symptoms induced by rCTV were identical to those induced by the parental T36 isolate in all indicator plants examined.

The rCTV appeared to replicate and move similarly to the wild-type parental virus in infected plants. Levels of accumulation in different parts of plants were similar, based on ELISA (data not shown). Figure 5 shows a comparative Northern blot hybridization of dsRNA from rCTV and wild-type CTV T36-infected plants. The levels and patterns of dsRNA appeared to be identical. CTV populations normally contain a range of dRNAs. To examine the development of new dRNAs, a series of rCTV-infected plants were examined in trees maintained in the greenhouse 9 months and 1 year after inoculation. No dRNAs were detected from the citrus plants infected with the rCTV (data not shown).

The parental virus population was known to be transmitted by aphids, but with a relatively low efficiency (Powell *et al.,* 1999). To examine whether the rCTV retained the ability to be aphid transmitted, the brown citrus aphid [*Toxoptera citricida* (Kirkaldy)] was used to transmit the rCTV and the parental wild-type virus. Both the wild-type CTV T36 and the rCTV were transmitted by the brown citrus aphid.

Certain cytopathological alterations that include the accumulation of virus aggregates and typical vesicular structures in phloem tissue are characteristic of CTV and other *Closteroviridae* (Bar-Joseph *et al.,* 1979a). The ultrastructure of plants infected by the rCTV or parental population was compared. A cross section of a marginal leaf vein of a Mexican lime plant at 30 dpi with rCTV showed the accumulation of virus particles (V), aggregates of p-protein (pP), amorphous inclusion bodies (aI), and vesicles (Ves) (Fig. 6) typical of CTV infections (Bar-Joseph *et al.,* 1979a).

DISCUSSION

The artificial life cycle of CTV, from tree to bacterium to tree, was completed with the ability to infect citrus trees with rCTV. CTV has been one of the more difficult viruses for which to produce a manipulatable genetic system. The lability of its virions makes the virus difficult to purify and reduces the specific infectivity of purified virion preparations. The large size of the RNA genome (~20 kb) greatly reduces its ability to infect protoplasts and plants, particularly with RNA as inoculum. Its limited host range, which is restricted to woody hosts and its further limitation to phloem-associated tissue in those plants, makes successful inoculation of plants difficult. However, the economic losses caused by this virus and the substantial threats to existing citrus industries demand development of a genetic system that could allow understanding and management of these diseases.

Although an infectious cDNA clone was produced that allowed manipulation of the virus *in vitro* and examination of the virus replication in protoplasts (Satyanarayana *et al.,* 1999), initial efforts to use RNA transcripts as inoculum failed to infect plants directly and infected far too few protoplasts to produce sufficient inoculum to infect plants. However, several key observations concerning the infection of protoplasts with CTV led to the amplification procedure of successively passaging the virions through protoplasts, which resulted in sufficient amounts of progeny virions for inoculation of small citrus trees. First was the observation that larger RNAs appear to be proportionally more difficult to get into protoplasts than smaller RNAs, and very large RNAs approach impossibility. Second was that virions were substantially more infectious than RNA. Third was that virions in crude sap were highly infectious. We previously showed that nonencapsidated viral RNA in sap was not infectious to protoplasts (Satyanarayana *et al.,* 2000). Using GFP as a reporter, we found that the increase in virus accumulation levels using different types of inocula (RNA, purified virions, or crude sap) largely resulted from increases in the proportion of protoplasts infected. This understanding led to the amplification of the low level of virions produced in primary protoplasts infected with RNA transcripts to very high levels in subsequently infected protoplasts using the highly infectious crude sap as inoculum. These highly infected batches of protoplasts produced relatively large amounts of progeny virions, which were sufficient to mechanically inoculate citrus trees by slashing. Other large or low-titer viruses, of which there are difficulties in infecting large proportions of protoplasts, might be amplified similarly. The relatively large

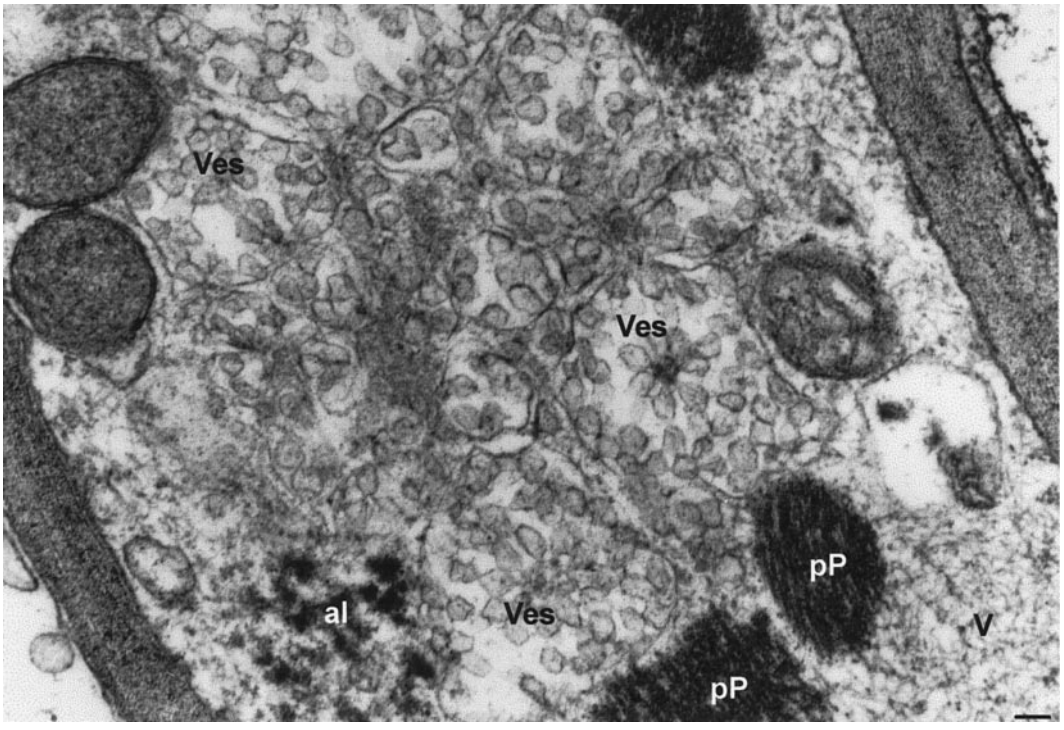

FIG. 6. Electron micrograph showing the ultrastructure of Mexican lime cells infected with rCTV at 30 dpi. Accumulation of large masses of virus particles (V), aggregates of p-protein (pP), amorphous inclusion bodies (al), and typical vesicles (Ves) in a phloem parenchyma cell. Bar, 100 nm.

amounts of amplified virions should be sufficient to inoculate plants by other procedures, for example, membrane feeding or direct protoplast feeding of insects for transmission. Even for routine experiments in protoplasts, inoculation with virions after amplification can be preferable to inoculation with RNA transcripts because the virus replicates in a much higher proportion of virion-inoculated protoplasts.

In retrospect, based on the size of full-genomic CTV, it is amazing that we were able to detect replication of the rCTV in protoplasts using *in vitro*-produced RNA transcripts as inoculum (Satyanarayana *et al.,* 1999), because the first positive results were Northern hybridization blots with less than one-tenth the intensity of those routinely observed now. A normal batch of protoplasts consists of approximately one million. Using improved inoculation procedures for the GFP-marked virus, we estimated that approximately 0.01% of protoplasts exhibited fluorescence. That would calculate to about 100 infected protoplasts. In the first successful experiments we might have observed replication in as few as 10 infected protoplasts per batch. This emphasizes the difficulty of working with large RNA viruses in plant systems.

The analysis of plants infected with the rCTV from pCTV9 indicates that the ~20-kb genome of CTV was reproduced from RNA to cDNA and back to RNA with enough fidelity to maintain the biological characteristics of the parental virus. Based on present observations, we cannot distinguish plants infected with rCTV and parental virus. The rCTV appears to replicate, move, induce symptoms, and be aphid transmitted similar to the parental virus.

Fulfilling Koch's postulates for the cause of disease has been difficult for viruses that exist only in complex populations. Citrus trees can be vegetatively propagated for hundreds of years, providing long periods for complex CTV populations containing a myriad of genomic RNAs and dRNAs to develop. The same is true for complex mixtures of closteroviruses in grape (Boscia *et al.,* 1995; Choueiri *et al.,* 1996), pineapple (Hu *et al.,* 1996), and cherry (Jelkmann *et* al., 1997) diseases. It is now possible to analyze the contribution of individual components of a population to disease production and to reconstruct populations from known components. In this report we were able to compare the "pure" culture of rCTV with that of the parental population. Although the parental isolate T36 appears more uniform than most CTV populations, consisting primarily of one master genotype (quasispecies), small amounts of a disparate T30-like sequence (Albiach-Martí *et al.,* 2000a) also occur (Hilf *et* al., 1999; Ayllón *et al.,* 2000) along with different dRNAs in different T36 isolates. Based on our initial analysis, the pure culture and the wild-type population have similar phenotypes in a range of indicator hosts. We could distinguish no biological contributions resulting from the minor genotypes and dRNAs of the parental population, suggesting that the major genotype of this isolate is the primary deter-

minant of the population phenotype. The *in vitro* genetic system of CTV now should allow the creation of mutants and hybrids between mild and severe genotypes, that would allow the mapping of specific disease determinants of T36 and other CTV strains to individual genes or nucleotides.

MATERIALS AND METHODS

Virus isolate and indicator plants

CTV (isolate T36) was maintained in Alemow and Mexican lime plants in a greenhouse at CREC (Lake Alfred, FL). Duncan grapefruit, Etrog citron, Madam vinous sweet orange, Mexican lime on Alemow rootstock, rough lemon, sour orange, Valencia sweet orange grafted to sour orange rootstocks, and Volkamer lemons were maintained in a greenhouse at USDA-ARS (Orlando, FL) and CREC (Lake Alfred, FL).

cDNA constructs, *in vitro* transcription, transfection of *N. benthamiana* protoplasts, and RNA analysis

Isolation of mesophyll protoplasts from *N. benthamiana* and polyethylene glycol-mediated transfection were previously described (Navas-Castillo *et al.,* 1997; Satyanarayana *et al.,* 1999). Freshly prepared capped *in vitro* transcripts from *Not*I-restriction enzyme linearized pCTV9 and its derivative mutants were used directly for inoculation of $\sim 1 \times 10^6$ protoplasts as described (Satyanarayana *et al.,* 1999). Total nucleic acids were extracted from protoplasts 3 and/or 4 dpi and analyzed by Northern blot hybridization as described in Satyanarayana *et al.* (1999). The dsRNA from Mexican lime plants infected with wild-type CTV T36 and rCTV was prepared according to Dodds and Bar-Joseph (1983). The 3′-terminal 900 nts of CTV T36 in pGEM-7Zf (Promega, Madison, WI) were used to make either positive- or negative-stranded RNA-specific riboprobes with digoxigenin-labeled UTP using either SP6 or T7 RNA polymerase.

Serial passage of rCTV in *N. benthamiana* protoplasts

N. benthamiana protoplasts were inoculated with freshly prepared *in vitro*-produced RNA transcripts from pCTV9 as described previously (Satyanarayana *et al.,* 1999). Protoplasts were harvested at 4 dpi and divided into two halves. One half was used to analyze the accumulation of CTV RNAs by Northern blot hybridization and the other half was stored at −70°C for subsequent transfer to the next batch of protoplasts. For transfer, the frozen protoplasts were suspended in 100 μl of 40 mM sodium phosphate buffer, pH 8.2, and kept on ice for 20–30 min with gentle intermittent mixing. The sap was clarified by centrifugation at 3000 rpm for 3 min and 60–75 μl of supernatant was used to inoculate a next batch of protoplasts. The crude sap containing virions (rCTV) was passaged serially seven to 10 times through protoplasts. Total protein from the phenolic phase of protoplasts at 4 dpi was acetone precipitated, separated on a 12.5% polyacrylamide/SDS gels, and analyzed by Western immunoblots as described by Towbin *et al.* (1979) using the MCA13 monoclonal antibody (Permar *et al.,* 1990). The rCTV virions, which were passaged four to 10 times through *N. benthamiana* protoplasts, were used to inoculate citrus plants.

Mechanical inoculation of citrus plants with rCTV

The frozen (−70°C) protoplasts inoculated with CTV9 were suspended in 125 μl of either phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBS-T) or 40 mM sodium phosphate buffer, pH 8.2, gently mixed by tapping, and incubated on ice for 20–30 min. The crude sap was centrifuged at 3000 rpm for 3 min and the supernatant, which contained rCTV virions, was used as the inoculum. Additionally, rCTV virions were partially purified from clarified extracts of serially passaged protoplasts using a 60% sucrose cushion as described previously (Hilf *et al.,* 1995). Slash inoculation was performed by placing several droplets (~5 μl) of crude sap extracts along the defoliated stems followed by a single cut (4–5 cm) along the drop line and a series of perpendicular slash cuts 2–3 mm apart. The slashed stems were protected from direct exposure to sunlight by wrapping with either parafilm or paper and plants incubated in controlled-environment plant growth chambers or in a greenhouse. One week after inoculation the plants were pruned and fresh shoots 4–8 weeks after inoculation were tested by ELISA according to Bar-Joseph *et al.* (1979b).

Aphid transmission

The aphids were reared on healthy Carrizo citrange seedlings (*Poncirus trifoliata X C. sinensis*) that were resistant to CTV, after which they were moved to new growth of Alemow plants infected with rCTV and fed for periods ranging from 3 to 15 h to acquire the virus. The aphids (20 to 30) were then brush transferred from the donor plant to young Mexican lime seedlings with terminal flush and allowed to feed for 24 h. The plants were then sprayed with an insecticide to kill all aphids and incubated in a controlled-environment plant growth chamber.

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