Infection of Citrus Plants with Virions Generated in *Nicotiana benthamiana* Plants Agroinfiltrated with a Binary Vector Based *Citrus tristeza virus*

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ABSTRACT. *Citrus tristeza virus* (CTV) of the *Closteroviridae* family has a plus-stranded RNA genome of approximately 20 kilobases. Purified virion RNA and *in vitro*-generated RNA transcripts from an infectious cDNA clone failed to infect citrus plants, although both RNAs initiated low levels of infection in the mesophyll protoplasts of *Nicotiana benthamiana* and citrus. Infection of citrus plants required successive protoplast passages of progeny virions from transcript-inoculated *N. benthamiana* protoplasts to maximize the virus titer. To improve the infection efficiency, we engineered a full-length CTV cDNA in a binary vector (pCAMBIA) that contained a 35S promoter with a duplicated enhancer in the 5' end, a ribozyme and nopaline synthase polyA signal at the 3' end, and which was transferred into *Agrobacterium tumefaciens* EHA105 strain for agroinoculation studies. Initial attempts at the direct agroinfection of citrus plants were not successful, but the DNA of this construct produced CTV specific RNAs and virions when transfected into *N. benthamiana* mesophyll protoplasts. Replication and virion formation were also observed in the agroinfiltrated leaves of *N. benthamiana,* and citrus plants became infected when inoculated with virions partially purified from the sap expressed from agroinfiltrated *N. benthamiana* leaves.

Index words. Closteroviridae, *Citrus tristeza virus*, binary vector, agroinfiltration.

Citrus tristeza virus (CTV) is a monopartite member of the *Closteroviridae* family and has a single-stranded positive-sense RNA genome of ~20 kilobases (kb). The 2000 nm long flexuous virions contain two coat proteins: the minor coat protein (CPm, 27 kDa) encapsidates ~3% of the genomic RNA at the 5' end and the major coat protein (CP, 25 kDa) encapsidates the remaining length of the RNA (10, 14). The two coat proteins result in the characteristic "rattlesnake" morphology of the virions (3). The genome is organized into 12 open reading frames (ORFs) (10, 14). The 5' proximal ORF 1a encodes a high molecular weight polyprotein that contains two papain-like proteases, a methyltransferase-like and a helicase-like domain, and ORF 1b encodes an RNA dependent RNA polymerase-like domain that is expressed through a +1 frameshift (10). The remaining 10 ORFs are not required for the replication of the genome (18) and are expressed through 3' co-terminal sgRNAs (8).

CTV has caused serious problems in citriculture worldwide. The term "tristeza", which means "sadness" in Spanish and Portuguese, describes the death of affected trees on sour orange rootstocks. Some isolates also cause stem pitting in sensitive scions, even on tolerant rootstocks. Stem pitting can reduce the size, yield, and marketability of the fruit. Some isolates induce no or very mild symptoms in the field or in experimental indicator plants and are termed "mild isolates". In some countries mild isolates have been used to cross protect against more "severe" isolates (11). Bio-indexing studies of aphid transmitted sub-isolates from sources infected with "mild" isolates of CTV revealed that some symptomatically mild isolates were mixtures harboring "severe" isolates as well (1, 11, 12). Unfortunately, most wild-type mild isolates fail to protect against

most severe isolates, and therefore, genetic engineering of protective mild isolates of CTV and their delivery into existing citrus trees offers promise in the management of CTV.

An infectious cDNA clone of the Florida decline isolate T36 provided a reverse genetic system to study the functional repertoire of its genes and their interactions with citrus to elicit diseases (18). However, for this system to be fully functional citrus plants must be infected with progeny RNA or virions derived from the cDNA clones. While infection of *N. benthamiana* leaf protoplasts with RNA transcripts from the infectious cDNA clone and the subsequent production of progeny virions have been demonstrated, efforts to directly inoculate citrus plants with RNA transcripts or virions isolated from transcript-inoculated protoplasts have not been successful. Tests with a GFP-labeled virus transcript indicated that only 0.01% of transcript-inoculated *N. benthamiana* protoplasts were infected and that the resultant amount of progeny virions was below the threshold needed to infect citrus plants (21). A more reliable method to infect citrus plants with cloned constructs based on concentration of virions amplified in *N. benthamiana* protoplasts has been developed (19) and amplification of the virus by successive passages through *N. benthamiana* protoplasts did allow successful inoculation of citrus plants (21). However, this method still requires successive passages of the virus in protoplasts, with a minimum of 3-6 months elapsing from initial protoplast inoculation to plant phenotypic evaluation. A single failure at any point in the multi-step process requires it to be begun again. Therefore, a more reliable and faster method to infect citrus plants with cloned constructs was needed.

Agroinoculation (2, 7, 17, 23) provides an alternative method with the potential to deliver CTV and its mutants directly into citrus plants.

This process could eliminate protoplast transfection and subsequent passage, and would greatly augment the infection of citrus plants and subsequent symptom evaluation. *Agrobacterium*-based binary vectors have been used routinely to transiently produce proteins in agroinfiltrated cells and for agroinoculation of plant viruses (4, 25).

Here we report the successful stepwise development of a binary vector containing a full length CTV that initiated replication in *N. benthamiana* protoplasts and *in planta* upon agroinfiltration of *N. benthamiana* leaves, but failed to infect citrus. Although the virus did not spread cell to cell in *N. benthamiana*, progeny virions isolated and concentrated from the infiltrated leaves established CTV infections in citrus plants.

MATERIALS AND METHODS

Construction of binary vector based CTV. A full-length cDNA clone of CTV, pCTV9 (18, 20), was used as a source plasmid for obtaining a full-length CTV cDNA in the *Agrobacterium tumefaciens* binary vector, pCAMBIA 1380 (15). The sequences of the oligonucleotide primers used in the construction of pCAM:CTV are listed in Table 1 and are based on the sequence of the CTV T36 infectious cDNA clone (20, GenBank accession No. AY170468). *Pfu*I turbo DNA polymerase (Stratagene, WI) was used for amplification of the DNA by polymerase chain reaction (PCR) and Vent DNA polymerase (New England Biolabs, MA) for overlap-extension PCR (9). The recombinant clone, pCAM:CTVdRNA, was generated in two steps. First, the 3' end of CTV containing the ribozyme was amplified using minus-sense oligonucleotide, CTV-M1 (containing the ribozyme sequence with an *Xba*I restriction site and CTV specific nts 19296-19267) and a plus primer, CTV-M2 (corresponding to CTV-specific nts 18313-18338 which con-

TABLE 1
SEQUENCE OF OLIGONUCLEOTIDES USED IN THIS STUDY

Oligonucleotides[1]	Sequence (5'-3')	Polarity	Position in CTV
CTV-M1[2]	AGCT<u>GAATTC</u>**GATCTCCTTTGCCCCAGAGATCAC AGTATAAGTACAGAC***TGGACCTATGTTGGC-CCCCCATAGGGACAG*	(-)	nt19267-19296
CTV-M2	AGCT<u>CTGCAG</u>*GTTAATACGCTTCTCAGAACGT-GTGG*	(+)	nt18313-18338
CTV-M3	AGCT<u>GAATTC</u>**GATCTCCTTTGCCCCAGAGATCAC**	(+)	
CTV-M4[3]	**GGAAGTTCATTTCATTTGGAGAGG***AATTTCA-CAAATTCAACCTGTTCGC*	(+)	nt 1-25
CTV-M5	*GCGAAGAGGTTGAATTTGTGAAATT***CCTCTC-CAAATGAAATGAACTTCC**	(-)	nt 25-1
CTV-M6	AGCT<u>CTGCAG</u>*CAACTAGTCCCCACATCCATCAT-AACCG*	(-)	nt2125-2098
CTV-M7	GGTAGGGACCTTCACACTGCGTTCGT	(+)	nt 999-1025
CTV-M8	AGCT<u>CTGCAG</u>*GGGTTTAAACAGAGTCAAACGGC-GAGTCTTAAG*	(-)	nt 11879-11851
M-706[4]	AGCG<u>CCATGG</u>**AACGAGCTATACAAGGAAAC**	(+)	
M-707	AGCA<u>GAGCTC</u>**TTACTCGCCTTCTTTTTCGA**	(-)	

[1]For CTV-M1-CTV-M8: CTV specific sequences are in italics, restriction enzyme sites are underlined, and the clamp nucleotides at the 5' end are in normal font.
[2]In CTV-M1 and CTV-M3, the nucleotides in bold represent ribozyme sequence and nucleotides at the 5' end and the CaMV 35S[2] promoter, respectively.
[3]For CTV-M4 and CTV-M5, the sequences in bold indicate 3'end sequences of the CaMV 35S[2] promoter and nucleotides in italics represent the CTV 5' end sequences, respectively.
[4]In M-706 and M-707 the nucleotides in bold represent the 5' and 3' end sequences of the TBSV p19 gene, respectively.

tained a *Pst*I restriction site at the 5' end) (Table 1). The amplified product was digested with *Xba*I and *Pst*I and ligated to *Spe*I and *Pst*I digested pCAMBIA 1380. In the second step, *Cauliflower mosaic virus* (CaMV) 35S[2] promoter was fused to the 5' end of CTV by overlap extension PCR and was ligated to the product of the first step. The CaMV 35S[2] promoter containing duplicated enhancer sequences used in this study was amplified from pKYLX-7 (22). The juxtaposition of the 3' end of the promoter with the 5' end of the CTV genome was carried out by a pair of overlapping primers CTV-M4 and CTV-M5 (Table 1) by two DNA amplification events followed by overlap extension PCR (9). The first amplification used a plus primer,

CTV-M3 (corresponding to the 5' end of 35S[2] promoter) with an *Eco*RI site, and minus primer, CTV-M5 (that contained nucleotides complementary to the 3' end of the 35S[2] promoter sequence and nt 25-1 of CTV) using pKYLX as the DNA template, while the second product was amplified by a plus primer, CTV-M4 (that contained nucleotides corresponding to the 3' end of the 35S[2] promoter and nt 1-25 of CTV) and the minus primer, CTV-M6 (complementary to CTV nts 2125- 2098 and containing a *Pst*I site) using pCTV9 as the DNA template. The amplified products were mixed and the final product was amplified using primers CTV-M3 and CTV-M6. The overlap extension product was digested with *Eco*RI and *Pst*I and ligated between

similarly digested plasmid from the previous step, that contained the 3' end of CTV and the ribozyme, to generate pCAM:CTVdRNA. The latter was digested with *Bgl*II and *Pst*I and ligated with a DNA fragment (~10 kb) obtained by similar digestion of pCTV-ΔCla333R (6) to generate the replicon pCAM:CTV 14R.

A full-length clone of CTV, pCAM:CTV947R, was generated from pCAM:CTVdRNA by three independent cloning events. First, the 5' CTV sequence in pCAM:CTVdRNA was extended in the 3' direction to include the unique *Bsu*36 I restriction site (nt 4430) by amplification of a DNA fragment (nt 1026-4430) using primers CTV-M7 (upstream of *Bgl*II site at nt 1029) and CTV-M8 (downstream of *Bsu*36 I site at nt 4430) (Table 1). The amplified product was digested with *Bgl*II and *Pst*I and ligated between similarly digested pCAM:CTVdRNA to generate pCAM:CTVdRNA-Bsu361 that contained unique *Pme*I and *Pst*I sites. Secondly, the plasmid pCAM:CTVdRNA-Bsu361 was digested with *Pme*I and *Pst*I and ligated with a DNA fragment (~5.4 kb) obtained by similar digestion of full-length CTV, CTV9R (18). The resulting construct, pCAM:dRNA-Bsu361-PmeI, which contained two unique restriction enzyme sites *Bsu*36 I and *Pme*I, was digested with the same enzymes and ligated with the DNA fragment (~7 kb) obtained by similar digestion of the full-length cDNA clone of CTV, CTV9R (18) to obtain the full-length CTV, pCAM:CTV947R. Nucleotide sequencing of the junctions between the 35S[2] promoter and the CTV 5' terminus, and the CTV 3' terminus and the ribozyme was performed at the Interdisciplinary Center for Biotechnology Research DNA sequencing core facility of the University of Florida, Gainesville, FL.

The p19 gene of *Tomato bushy stunt virus* (TBSV) (kindly provided by Dr. Herman Scholthof, Texas A&M University) was amplified using primers M-706 and M-707 (Table 1). The amplified product was digested with *Nco*I and *Sac*I and ligated to the similarly digested binary plasmid, pCAMBIA 1300 (GenBank accession no. AF234296), to place the TBSV sequence between the 35S promoter and the nopaline synthase (Nos) polyA signal to generate pCAM:p19. This construct was used as a suppressor of gene silencing.

Agroinfiltration of *N. benthamiana* leaves and inoculation of citrus plants. The DNA from pCAM:CTV14R or pCAM:CTV947R was used to transform *A. tumefaciens* EHA 105 cells, and the transformants were selected on LB plates containing the antibiotics Rifampicin (50 µg/ml) and Kanamycin (50 µg/ml). The transformants were grown overnight in LB liquid medium containing the antibiotic Kanamycin (50 µg/ml), 10 mM MES (pH5.8), and 20 mM acetosyringone. The culture was centrifuged at 6000 rpm and the resulting pellet was washed twice with 10 mM MES, pH 5.8, containing 10 mM $MgCl_2$ and suspended in the same buffer containing 150 mM acetosyringone and adjusted to a OD_{600} of 0.5-1.0. The infiltration of *N. benthamiana* leaves by the agrobacterium culture was carried out as described (25). A mixture of *A. tumefaciens* containing pCAM:CTV947R and *A. tumefaciens* containing pCAM:p19 (containing the silencing suppressor), were infiltrated into the abaxial side of *N. benthamiana* leaves. The concentration of the *A. tumefaciens* culture OD_{600} was adjusted between 1.0-1.5 in coinfiltration experiments. The infiltrated leaves were harvested 7-8 days post-infiltration (dpi), diced in 40 mM potassium phosphate buffer, pH 7.2, containing 5% sucrose and 10 mM DTT, squeezed through cheese cloth, layered over 1 ml of 70% sucrose and centrifuged at 38,000 rpm for 75 min at 4°C. Approximately 0.4 ml from the bottom of the sucrose cushion was discarded. Sub-

sequently, 3-4 fractions of 0.1 ml each were collected as described by Robertson et al. (16), and examined by ELISA (5) and serological specific electron microscopy (SSEM). The fractions rich in CTV virions were combined and were directly used to bark-flap inoculate *Citrus macrophylla* (Alemow) plants (21).

Transfection of *N. benthamiana* mesophyll protoplasts, isolation of total RNA and Northern blot analysis. The DNA from pCAM:CTV14R or pCAM:CTV947R (20 mg each) were transfected into *N. benthamiana* mesophyll protoplasts, essentially in a manner similar to transfections with transcripts (18). RNA transcripts from the pCTV-ΔCla333R replicon were generated and transfected as described previously (18).

Infiltrated leaves were harvested and were used immediately or stored at -70°C for subsequent isolation of nucleic acids. The leaves were ground in the Buffard buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 9.0, plus 2% SDS), and extracted twice with a mixture of phenol, chloroform and isoamyl alcohol, followed by precipitation with ethanol (13). Preparation of digoxigenin labeled RNA probes and Northern blot analysis procedures were as described (18).

ELISA and SSEM analysis. Double antibody sandwich indirect enzyme-linked-immunosorbent assays (DAS-I-ELISA) (5) used purified IgG from rabbit polyclonal antibody CTV-908 (1 µg/ml) for coating and a broadly reactive CTV Mab172 for detection (5, 16). Serological specific electron miscroscopy (SSEM) analysis was carried out as described earlier (18).

RESULTS

DNA of binary-vectors with cloned CTV initiates replication in protoplasts. Before the construction of full-length CTV in the binary vector pCAMBIA 1380 (Fig. 1A), we generated intermediate clones of CTV (Fig. 1B) that contained the required *cis*-elements for replication and tested their ability to be replicated in protoplasts. Initially a defective clone of CTV, pCAM:CTVdRNA (Figs. 1B, 2A), containing only the 5' and 3' regions of the infectious CTV cDNA clone between the 35S^2 promoter (at the 5' end) and the ribozyme (at the 3' end) was cloned into pCAMBIA 1380. Sequencing showed this construct had the 5' terminal nucleotide of CTV correctly juxtaposed with the transcription start site of the CaMV 35S^2 promoter (Fig. 2B). The pCAM:CTVdRNA contained CTV nts 1-2128 and 18313-19296 (which corresponds to the 5' non-translated region, 5' portion of ORF 1a, p23 ORF with its 5' non coding region and the 3' NTR), followed by the ribozyme sequence and the Nos polyA signal (the latter derived from the vector) (Fig. 2A). Thus, pCAM:CTVdRNA contained all the necessary *cis*-sequences necessary for replication of the genomic RNA and production of p23 sgRNA upon cotransfection with a helper replicon.

To determine if pCAM:CTVdRNA replicated in protoplasts, the DNA was mixed with RNA transcripts from the CTV RNA replicon, pCTV-ΔCla333R (6) (as the helper virus pCTV-ΔCla333R replicates in *N. benthamiana* protoplasts but produces no sgRNA) and transfected into *N. benthamiana* mesophyll protoplasts. Northern analyses of RNA extracted from transfected protoplasts hybridized with a plus-strand riboprobe specific to the CTV 3' end, showing that the DNA of pCAM:CTVdRNA initiated transcripts which replicated and produced the p23 sgRNA (Fig. 2C lane 1) in the presence of the helper replicon. These results suggested that the pCAM:CTVdRNA contained all necessary *cis*-signals for replication and could be used for the construction of full length CTV in the binary vector.

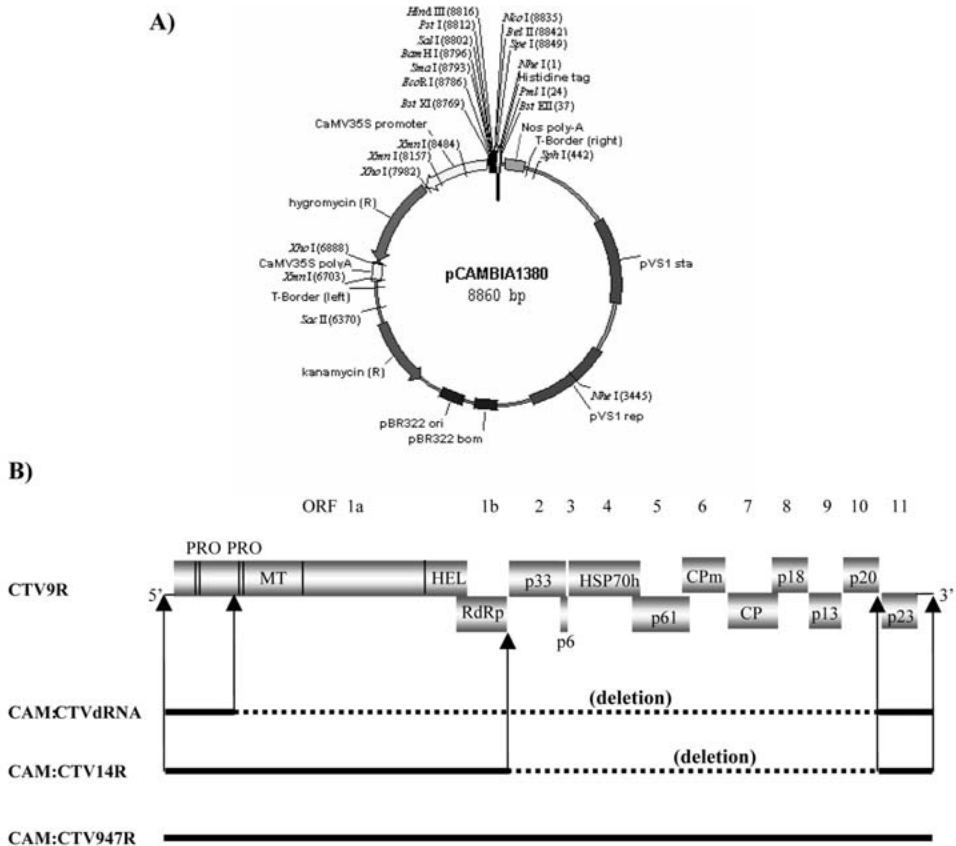As a next step we generated a DNA replicon of CTV, pCAM:CTV14R,

Fig. 1. A) Genomic map of the binary vector, pCAMBIA 1380 (15) with positions and coordinates of the useful markers and restriction enzymes indicated. B) Schematic representation of the genomic organization of wild-type CTV (CTV 9R) and CTV constructs generated in the binary vector, pCAMBIA1380. The open boxes indicate the ORFs and their translation products: PRO, papain-like protease, MT, methyltansferse-like; HEL, helicase-like domains and RdRP, RNA dependent RNA polymerase. The 5' and 3' at the ends represent the 5' and 3' non-translated regions, respectively. The solid and broken lines represent retained and deleted portions of the constructed CTV genomes, respectively, and the upward arrows indicate the corresponding genomic coordinates.

in the binary vector (Figs. 1B, 3A). This construct was similar to pCAM:CTVdRNA except it contained the complete replicase sequence (CTV ORF 1a and 1b) and would be competent for replication in inoculated protoplasts without a helper replicon. The replication competence of each of three independent clones of pCAM:CTV14R were tested in mesophyll protoplasts of *N. benthamiana.* Northern analysis of the total RNA isolated from the protoplasts 4 dpi demonstrated that the

pCAM:CTV14R DNA initiated replication, as observed by the production of genomic and sgRNAs (Fig. 3B).

Based on these results, we generated a full-length clone of CTV, pCAM:CTV947R (Figs. 1B, 4A) in the binary vector (see Materials and Methods). Northern analysis of total RNA isolated 4 dpi from transfected *N. benthamiana* protoplasts showed that three independent clones of pCAM:CTV947R, initiated replication and produced genomic and sg RNAs (Fig. 4B).
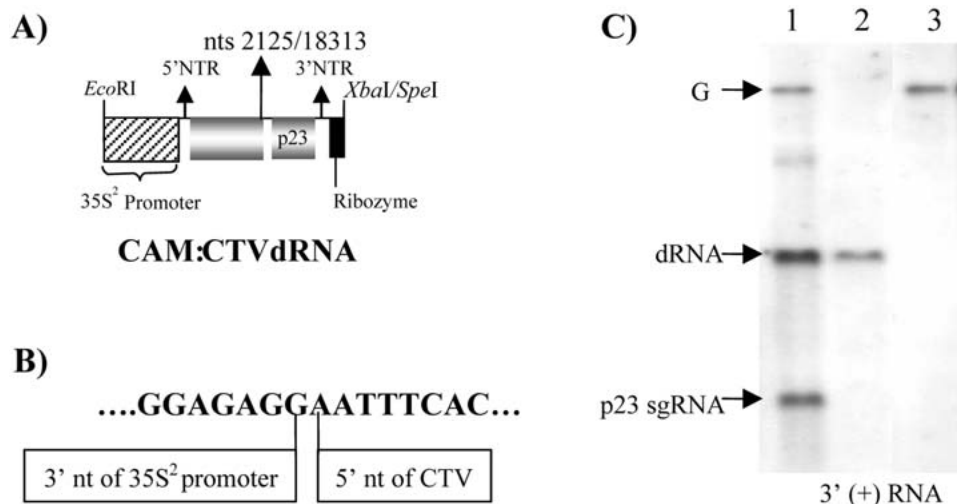
Fig. 2. A) Genome organization of the defective RNA construct pCAM:CTVdRNA. The open boxes represent coding portions of the CTV genome. Upward arrows indicate the coordinates where ORF 1A and the p23 ORF and nontranslated region are joined, and restriction enzyme sites of pCAMBIA (*Eco*RI and *Spe*I/*Xba*I) used in construction are indicated. Positions of the 35S² promoter and the ribozyme are shown. B) Nucleotide (nt) sequence of the junction of the 3' end of the promoter and the CTV 5' end showing the last nt (G) of the 35S² promoter and the 1st nt (A) of CTV. C) Northern blot analysis of total RNA isolated from the *N. benthamiana* mesophyll protoplasts four days post transfection with the DNA from: Lane 1) pCAM:dRNA plus RNA transcripts of pCTV-ΔCla333R, Lane 2) pCAM:dRNA and Lane 3) RNA transcripts of CTV RNA replicon, pCTV-ΔCla333R. The arrows indicate the genomic (G), defective (dRNA) and p23 subgenomic (sg) RNAs. The blot was hybridized with a digoxigenin labeled CTV 3' end riboprobe specific for the positive sense RNA (18).

Infection of *N. benthamiana* plants using binary-vector constructs of CTV in *A. tumefaciens*. To determine if pCAM:CTV14R could replicate *in planta,* a culture of *A. tumefaciens* EHA105 carrying pCAM:CTV14R was infiltrated into leaves of *N. benthamiana*. Northern hybridization analysis of total RNA extracted from the infiltrated leaves at 1, 2 and 4 dpi indicated replication, but the genomic and sgRNA signals were very low, which suggested very inefficient replication (data not shown). However, upon co-infiltration with a culture of *A. tumefaciens* carrying pCAM:p19 (which expresses a silencing suppressor gene of TBSV), infiltrated *N. benthamiana* leaves showed substantially higher levels of replication of pCAM:CTV14R genomic and subgenomic RNAs (Fig. 3C), demon-strating pCAM:CTV14R replication in both protoplasts and *in planta*.

Similarly, a culture of *A. tumefaciens* EHA 105 with the binary vector pCAM:CTV947R (which carries the complete CTV genome) was infiltrated into the leaves of *N. benthamiana* either singly or with *Agrobacterium* carrying pCAM:p19. Northern analysis of total RNA isolated from the infiltrated leaves at 3, 6 and 9 dpi demonstrated that CTV-specific genomic and sgRNAs were detected only when the TBSV p19 gene was used as a silencing suppressor (Fig. 4C).

Inoculation of citrus plants with binary-vector constructs of CTV. Repeated attempts to obtain replication of CTV from pCAM:CTV14R or pCAM:CTV947R (either singly or together with pCAM:p19) by infiltration of leaves
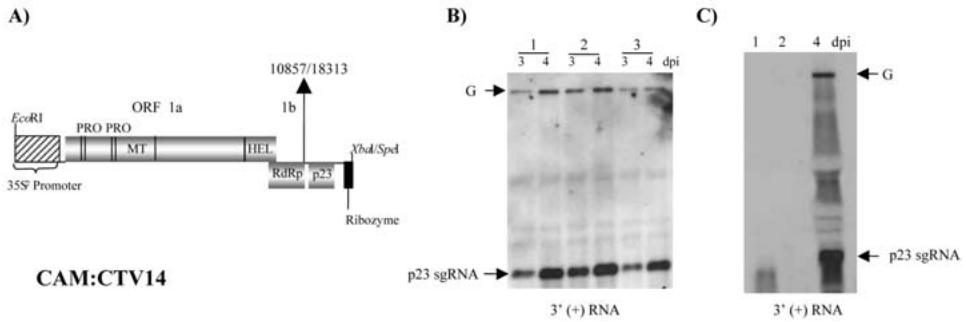
Fig. 3. A) Schematic representation of the CTV DNA replicon, pCAM:CTV14R. Details of the ORFs and their encoded products are as outlined in the legend to Fig. 1. B). Northern blot analysis of total RNA isolated 3 and 4 dpi from protoplasts of *N. benthamiana* transfected with DNA from three (1, 2 and 3) independent clones of pCAM:CTV14R. C). Northern blot analysis of total RNA isolated 1, 2 and 4 days post infiltration from the leaves of *N. benthamiana* agroinfiltrated with mixtures of *A. tumefaciens* EHA 105 transformed with pCAM:CTV14R and pCAM:p19. Positions of the genomic (g) and subgenomic (sg) RNAs are indicated by the arrows. Details of the hybridization and the probe used are outlined in the legend for Fig. 2.

or mechanical inoculation of the inner side of the bark flaps of young stems of Alemow by flooding with pCAM:CTV14R or pCAM:CTV947R *Agrobacterium* suspensions were unsuccessful. Either the constructs did not initiate replication or the level of replication in citrus leaves was too low to detect.

Inoculation of citrus plants with virions generated via agro-infection of *N. benthamiana*

leaves. Prior work demonstrated the infectivity of virions derived from an infectious cDNA clone of CTV which had been sequentially passaged in *N. benthamiana* proto-plasts (19). We conducted tests to determine if virions isolated from the infiltrated leaves of *N. benthamiana* also could be used to infect citrus plants. Fully expanded leaves from a large batch of *N. benthamiana* plants (10-12 plants) were agroinfil-
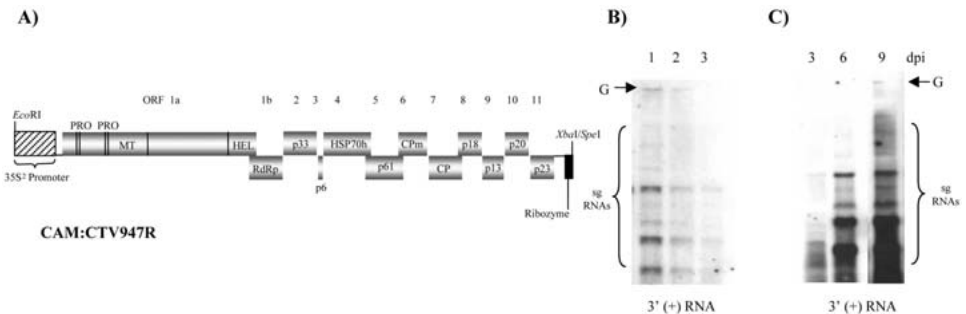

Fig. 4. A). Schematic of the construct, pCAM:947R, a full-length clone of CTV in the binary vector, pCAMBIA 1380. Details of the ORFs and the encoded products are as outlined in the legend for Fig. 1. Northern hybridization analysis of the total RNA from (B) protoplasts of *N. benthamiana* transfected with the DNA from three independent clones (1-3) of pCAM:947R, and (C) leaves of *N. benthamiana* co-infiltrared with mixture of *A. tumefaciens* EHA 105 containing pCAM:947R and pCAM:p19 and sampled at 3, 6 and 9 dpi. Positions of the genomic (G) and subgenomic (sg) RNAs are shown by the arrow and bracket, respectively. Details of the probe used for Northern analysis is outlined in the legend for Fig. 2.

trated, and virions were extracted from the infiltrated leaves and concentrated by centrifugation onto a 70% sucrose cushion. The virions were inoculated into Alemow stems by bark-flap inoculation (16). The virion preparation contained a number of CTV-like, full-length particles along with many smaller, apparently broken particles (not shown). Three of four inoculated plants developed classic CTV symptoms of leaf cupping and vein clearing 8-10 weeks post inoculation (Fig. 5A, B). DAS-I-ELISA revealed high titers of CTV (OD$_{405}$ >3.5) in bark and leaves of the three symptomatic plants, and these extracts contained CTV virions readily observed by SSEM (Fig. 5C).

DISCUSSION

In the present investigation we selected the *Agrobacterium*-mediated (24) delivery of CTV into citrus plants. Although, successful delivery of plant viruses through agroinfiltration of plants has been achieved previously (2, 7, 17 23, 25), direct infection of citrus plants with CTV constructs by agroinoculation had not been accomplished. In this study, we built a binary vector capable of initiating infection of CTV in *N. benthamiana* protoplasts and leaves. Infection of citrus plants with virions harvested from agroinfected *N. benthamiana* leaves circumvents the time and labor problems associated with making multiple sequential passages in protoplast to increase virion titers to threshold levels high enough for successful inoculation of citrus plants. The failure to infect citrus plants directly via agroinoculation apparently is a vector related problem and not a problem with the construct. It is possible that the EHA 105 strain of *A. tumefaciens* that we used may not be effective for infection of citrus. Further tests with other agrobacterium strains are warranted to determine if the problems are associated with specific species of *Agrobacterium*.

The process of agroinfiltration of pCAM:CTV947R into *N. benthamiana* represented an improvement in virion production for inoculation of citrus plants. This procedure can replace the time consuming and inconsistent system of inoculation of citrus plants by virions produced by amplification through serial passage in protoplasts. However, direct agroinoculation of citrus trees with these CTV constructs is still the ideal goal. Inocula from both proto-
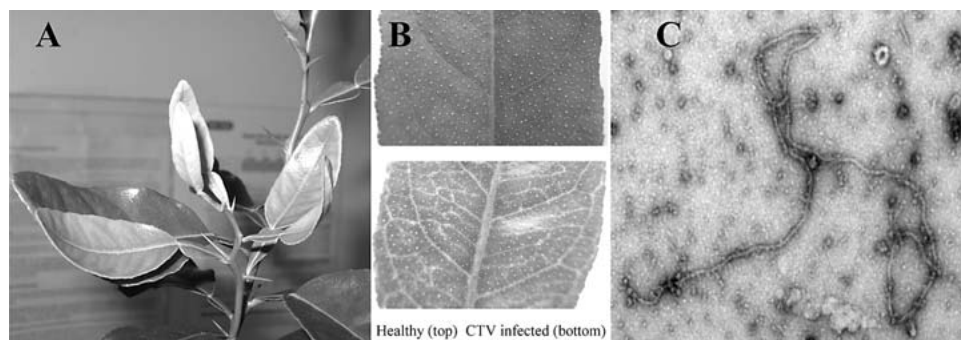

Healthy (top) CTV infected (bottom)

Fig. 5. Alemow plants showing (A) characteristic leaf cupping and (B) vein clearing symptoms (close-up comparison of infected, top panel versus healthy, bottom panel) associated with CTV infection eight weeks after inoculation with virions isolated from *N. benthamiana* leaves agroinfiltrated with pCAM:947R in *A. tumefaciens*. C). Serological specific electron microscopic analysis of the extract from the infected Alemow bark tissue showing flexuous filamentous CTV-like particles

plast passage and *N. benthamiana* leaves is limited to wild-type viruses and mutants that form stable virions, and multi-component constructs require a high multiplicity of infection. Direct agroinoculation of citrus trees would allow the examination in citrus trees of mutants with defective virions or with genomes divided among different RNAs. Yet, for now, agroinoculation of *N. benthamiana* as source of virus inoculum is the quickest procedure to introduce new CTV constructs into citrus plants.

ACKNOWLEDGMENTS

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