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Agroinoculation of *Citrus tristeza virus* Causes Systemic Infection and Symptoms in the Presumed Nonhost *Nicotiana benthamiana*

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Citrus tristeza virus **(CTV) naturally infects only some citrus species and relatives and within these it only invades phloem tissues. Failure to agroinfect citrus plants and the lack of an experimental herbaceous host hindered development of a workable genetic system. A full-genome cDNA of CTV isolate T36 was cloned in binary plasmids and was used to agroinfiltrate *Nicotiana benthamiana* leaves, with or without coinfiltration with plasmids expressing different silencing-suppressor proteins. A time course analysis in agroinfiltrated leaves indicated that CTV accumulates and moves cell-to-cell for at least three weeks postinoculation (wpi), and then, it moves systemically and infects the upper leaves with symptom expression. Silencing suppressors expedited systemic infection and often increased infectivity. In systemically infected *Nicotiana benthamiana* plants, CTV invaded first the phloem, but after 7 wpi, it was also found in other tissues and reached a high viral titer in upper leaves, thus allowing efficient transmission to citrus by stem-slash inoculation. Infected citrus plants showed the symptoms, virion morphology, and phloem restriction characteristic of the wild T36 isolate. Therefore, agroinfiltration of *Nicotiana benthamiana* provided the first experimental herbaceous host for CTV and an easy and efficient genetic system for this closterovirus.**

Citrus tristeza virus (CTV), a member of genus *Closterovirus*, family *Closteroviridae*, is the most destructive viral pathogen of citrus and one of the more economically important plant viruses. In nature, its host range is restricted to species of a few genera within the subfamily *Aurantioideae*, and within infected plants, the virus invades only phloem tissues. CTV has been experimentally transmitted to *Passiflora gracilis* and *P. caerulea* (Müller et al. 1974; Roistacher and Bar-Joseph 1987), two perennial vines, but attempts to transmit it to herbaceous and other nonrutaceous woody species, including *Nicotiana benthamiana* and other *Nicotiana* species, were unsuccessful (Müller and Garnsey 1984; our unpublished results). Almost 100 million trees propagated on sour orange rootstocks died worldwide from tristeza epidemics, and presently, many millions more propagated on decline-tolerant rootstocks are debilitated by stem pitting–inducing CTV isolates (Bar-Joseph and Dawson 2008; Moreno and Garnsey 2010).

CTV virions (2,000 × 10 to 12 nm) are composed of a single-stranded, positive-sense genomic RNA (gRNA) of about 20 kb and two coat proteins of 25 (CP) and 27 (CPm) kDa that encapsidate about 97 and 3% of the gRNA, respectively (Bar-Joseph and Lee 1989; Gowda et al. 2009; Satyanarayana et al. 2004). The CTV gRNA is organized into 12 open reading frames (ORF) and nontranslated regions (NTR) of 107 and 273 nt at the 5′ and 3′ termini, respectively (Karasev et al. 1995). ORF 1a and 1b, encompassing the 5′ half of the genome, encode replicase-related proteins that are translated from the gRNA. The 349-kDa protein encoded by ORF 1a contains two papainlike protease domains plus methyltransferaselike and helicaselike domains, and its translation is thought to occasionally continue through the polymeraselike domain encoded by ORF 1b by a +1 frameshift. The ten 3′-proximal ORF encode proteins p33, p6, p65, p61, p27, p25, p18, p13, p20, and p23, which are expressed via 3′ coterminal subgenomic RNAs (sgRNAs) (Hilf et al. 1995), promoted by internal controller elements (Gowda et al. 2001). Proteins p65, p61, p27, and p25 are part of a group involved in virion assembly and movement that is conserved among closteroviruses (Dolja et al. 2006; Gowda et al. 2009; Satyanarayana et al. 2000, 2004; Tatineni et al. 2010). The function of p33, p18, and p13 is presently unknown, and deletion mutants lacking these genes are able to systemically infect some citrus species (Tatineni et al. 2008). Proteins p25, p20, and p23 have been shown to act as silencing suppressors in *N. benthamiana* and *N. tabaccum* plants (Lu et al. 2004).

Setting up a genetic system based on an infectious cDNA clone was a challenging objective because i) the large size of the CTV genome hindered preparation of full-length cDNA clones and, then, of intact RNA transcripts for inoculation; ii) although citrus can be mechanically inoculated by slashing citrus stems with CTV virions (Garnsey et al. 1977), all attempts to mechanically inoculate them with virion RNA or RNA transcripts from a cDNA clone were unsuccessful (Satyanarayana et al. 2001); and iii) we lacked an herbaceous host for experimentation. Satyanarayana and associates (1999) developed a full-length cDNA clone of the CTV isolate T36 (CTV-T36) genome, from which they synthesized in vitro RNA transcripts that infected *N. benthamiana* protoplasts and produced normal CTV virions. However, due to the large size and fragility of the RNA transcripts and the difficulty of inoculating proto-

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plasts with such large RNAs, the protoplast infection rate was low (about 10^{-4}) and the amount of virions obtained was insufficient for successful infection of citrus plants by mechanical inoculation. This made necessary virion amplification by five to six cycles of protoplast inoculations to show that these virions systemically infected citrus plants and were able to incite symptoms identical to those of the wild T36 isolate (Satyanarayana et al. 2001). These limitations make this genetic system very tenuous. The transfer process often failed.

Viral genomes have been efficiently delivered into plant cells by infiltrating tissues with *Agrobacterium tumefaciens* cultures carrying appropriate binary plasmids with a full-length cDNA of the virus genome (Grimsley et al. 1986). This procedure has been successfully used with a citrus-infecting virus (Vives et al. 2008) and with several phloem-limited viruses (Leiser et al. 1992; Stephan and Maiss 2006), including members of the family *Closteroviridae* (Chiba et al. 2006; Liu et al. 2009; Wang et al. 2009). However, previous attempts to agroinoculate alemow (*C. macrophylla* Wester) or Mexican lime (*C. aurantifolia* (Christ.) Swing.) plants with binary plasmids carrying a cDNA clone of CTV-T36 were unsuccessful (Gowda et al. 2005; our unpublished data). Therefore, trials were aimed at developing a genetic system based on agroinfiltration of *N. benthamiana* plants as intermediate host to produce enough CTV virions to mechanically infect citrus plants by stem-slash inoculation. In preliminary experiments citrus plants were successfully infected with purified virion extracts from agroinfiltrated *N. benthamiana* leaves harvested at 7 to 8 days postinfiltration (dpi) (Gowda et al. 2005); however, erratic results in different experiments made the procedure unreliable. In this work, we agroinfiltrated *N. benthamiana* leaves with several binary plasmids carrying a cDNA of the CTV-T36 genome and different silencing suppressors and performed a timecourse analysis of CTV accumulation in those leaves. Unexpectedly, we found that agroinoculated plants of this species, presumed to be 'nonhost,' were systemically invaded by CTV-T36 with high viral titers both at the inoculated and upper leaves, in which typical disease symptoms were observed. The virus was eventually found also in nonphloem tissues, reaching an even higher viral load in the upper leaves. Citrus plants mechanically inoculated with virions produced in *N. benthamiana* were systemically infected, showed the symptoms characteristic of the wild CTV-T36 isolate, and kept the virus restricted to the phloem.

RESULTS

Construction, stability, and in planta functionality of binary plasmids expressing the CTV genome.

Previous attempts to infect citrus plants directly by biolistic or mechanical inoculation of full-genome cDNA clones of CTV-T36 were unsuccessful (Gowda et al. 2005; our unpublished data). Since this CTV genotype was known to replicate in *N. benthamiana* protoplasts, we tried to develop a workable genetic system based on agroinfiltration of these clones after subcloning them in appropriate binary plasmids. For this purpose, we constructed the pUC-based plasmid p35SC31 carrying a full-genome cDNA of CTV-T36 (clone pCTV9) followed by a ribozyme, located between the 35S promoter of the *Cauliflower mosaic virus* (CaMV) and the nopaline synthase terminator (NOS-t) (Fig. 1C; Table 1). A minireplicon having the elements essential for replication (p35SΔCla9) was also obtained from p35SC31, as described by Satyanarayana and associates (1999) (Fig. 1C and D; Table 1). These constructs were designed to obtain, in planta, a CTV gRNA or a minireplicon with the precise 5′ and 3′ termini after subcloning the expression cassette in a binary plasmid and using this for plant agroinfiltration.

A major problem with CTV cDNA clones is the presence in ORF 1a of sequences toxic for bacteria, which impair their normal growth and cause plasmid reorganizations, particularly when using high copy–number plasmids. Moreover, the first cDNA clone of CTV-T36 (pCTV9) that had an accidental frameshift mutation at position 3,732 (Fig. 1D) had these problems but a restored version thereof (pCTV9R) was even more toxic (Satyanarayana et al. 2003). We tried first to use pBIN19, a low-copy binary vector (10 to 20 copies per cell), but only the constructs derived from pCTV9 (pBINCTV9 and pBINΔCla9) could be assembled. Agroinfiltration of *N. benthamiana* leaves with *A. tumefaciens* EHA105 or COR308 (Hamilton 1997) cultures transfected with these constructs resulted in a faint expression of the CTV minireplicon or the gRNA (data not shown) at 5 days postinfiltration (dpi) only when coinfiltrated with a silencing suppressor (Fig. 2A). Expression of CTV gRNA decayed quickly and did not give a stable infection (data not shown).

To improve the stability of CTV cDNA clones we i) inserted a plant intron to interrupt the toxic sequence in the CTV genome and ii) used a bacterial artificial chromosome (BAC) that produces one to two copies per cell and is capable of maintaining large eukaryotic cDNA fragments (Hamilton 1997). The intron 2 of the *ST-LS-1* gene of potato was inserted at positions 3,652 or 3,744 in ORF 1a of the minireplicon p35SΔCla295R, a restored version of p35SΔCla9 without the frameshift mutation (Fig. 1D; Table 1), following a strategy that conserves the optimal consensus sequence for splicing in plants (Johansen 1996; Vancanneyt et al. 1990). Clones carrying the intron at either position (p35SΔCla295Rint) showed similar stability in *Escherichia coli* JM109 grown in Luria Bertani (LB) agar-ampicillin, that formed colonies of 1.1 to 1.3 mm in diameter 1 day after plating, in contrast to the 0.7 to 1.4 mm in diameter of the colonies produced by the parent clone without intron (p35SΔCla295R) after 2 days of incubation. The toxicity problem was eliminated and stabilization was observed in the full-genome cDNA clone after intron insertion (p35SC31Rint) (Fig. 1D; Table 1), resulting in 1.2- to 1.4-mm colonies 1 day after plating, compared with the 2 to 3 days required to obtain a similar size with the parent clone (Satyanarana et al. 2003). Subcloning of the minireplicon p35SΔCla295R or the full-genome clones p35SC31 and p35SC31Rint into a BAC yielded BBACΔCla295R, BBAC31, and BBAC31Rint plasmids, respectively (Fig. 1E; Table 1). BBAC31R was obtained by ligation of the 3′ terminal *Sma*I/*Not*I fragment of p35SC31 into BBACΔCla295R (Fig. 1; Table 1). All of them were stable in *E. coli* DH10B grown on LB agar-kanamycin and formed visible colonies after 1 day, with BBAC31Rint producing larger 1-day colonies (1.5 to 1.8 mm) than BBAC31R (0.7 to 1.0 mm).

The functionality of the new constructs in planta in comparison with others based on the high copy–number plasmid pCAMBIA1380 (50 to 100 copies per cell) was tested by agroinfiltrating *N. benthamiana* leaves with *A. tumefaciens* COR308 cultures transfected with plasmids BBACΔCla295R or pCAMΔCla14R that carried a minireplicon of CTV-T36 (Table 1; Fig. 1), with or without expression of a silencing suppressor (Voinnet et al. 2003). Results with the two plasmids were similar; replication of viral RNA in agroinfiltrated leaves was detected by Northern blot at 5 dpi and by reverse transcription-polymerase chain reaction (RT-PCR) at 3 and 5 dpi, with an apparent RNA increase at the latter sampling time (Fig. 2A and C). Expression of a silencing suppressor increased CTV RNA accumulation in both cases (Fig. 2A). Similar experiments delivering, in planta, the full-genome plasmids BBAC31 and BBAC31Rint showed that they were also functional and allowed transient expression of CTV gRNA in agroinfiltrated leaves, albeit plasmid with an intron (BBAC31Rint) showed higher

viral RNA accumulation (Fig. 2B). The correct RNA splicing was verified by RT-PCR analysis of the viral progeny from BBAC31Rint using primers encompassing the intron region in the CTV cDNA. While PCR amplification from plasmids BBAC31Rint and BBAC31 yielded fragments of 622 and 433 bp, respectively, RT-PCR amplification using double-stranded (ds)RNA from CTV-infected citrus or total RNA extracts (RNAt) from *N. benthamiana* leaves agroinfiltrated with BBAC31Rint yielded a unique fragment of 433 bp (Fig. 2B), indicating efficient removal of the 189-nt intron. Sequencing of the 433-nt fragment amplified from *N. benthamiana* confirmed that the original viral sequence had been restored.

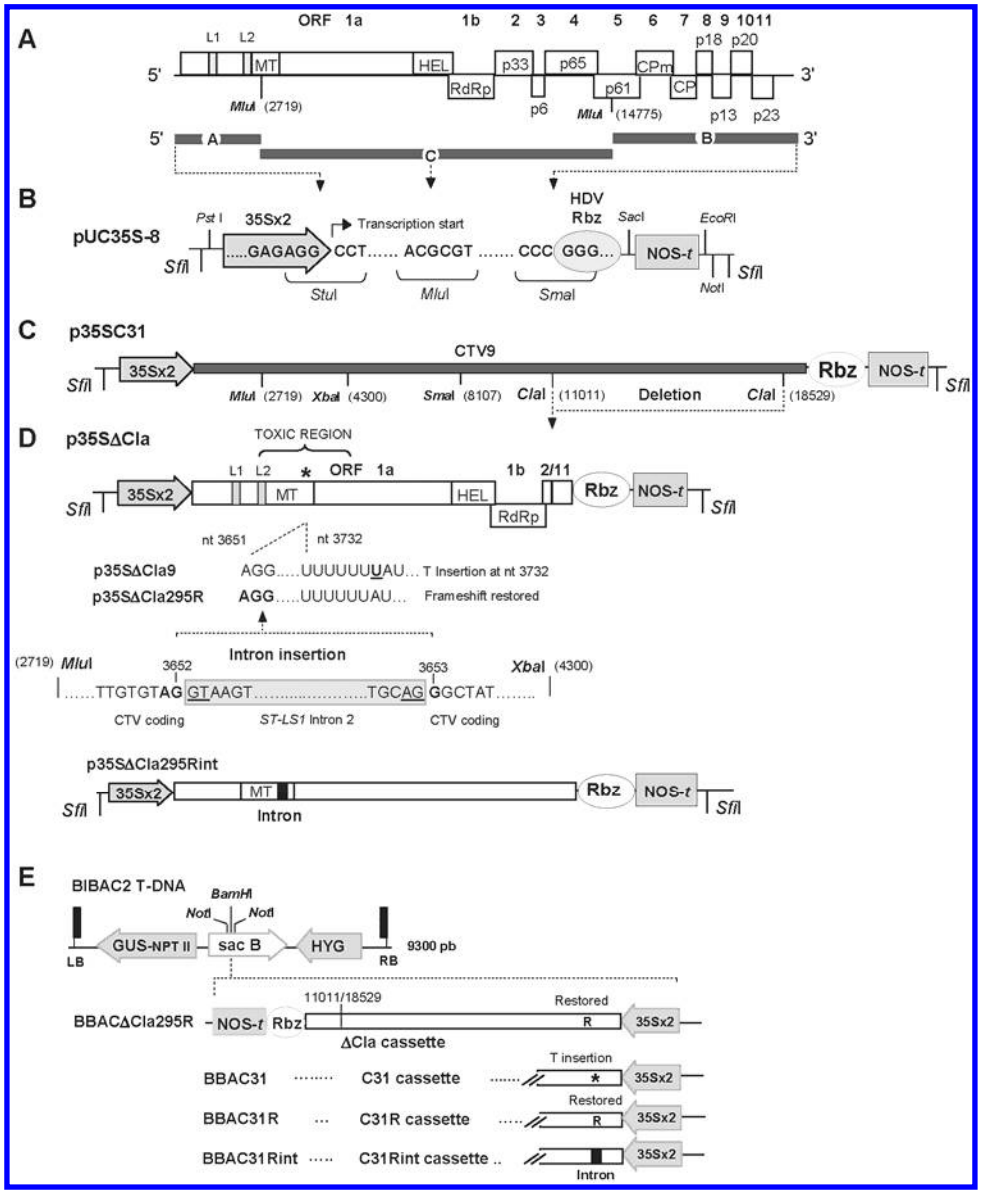

Fig. 1. Outline of the constructs used in this study. **A,** Genome organization of the *Citrus tristeza virus* (CTV) genomic (g)RNA. The boxes indicate open reading frames (ORF) and their corresponding translation products. L1 and L2 mean the two leader papainlike proteases; MT, methyltransferase; HEL, helicase; RdRp, RNA-dependent RNA polymerase; and CPm and CP, the minor and major coat proteins. Restriction sites used for subcloning and their position in the gRNA are indicated below. DNA products obtained by polymerase chain reaction amplification are shown below the genome map. **B,** Partial map of the pUC35S-8 vector containing a double-enhanced 35S promoter (35S × 2) of the *Cauliflower mosaic virus* (CaMV), the *Hepatitis delta virus* ribozyme (Rbz) and the nopaline synthase terminator (NOS-*t*). Relevant sequences for transcription start at the junction of the promoter and the viral sequence, for a precise 3′ end at the junction with the ribozyme, and restriction sites used for the subcloning steps are indicated. Dotted arrow lines indicate the cloning strategy to assemble the three cDNA segments encompassing the full CTV genome into the pUC35S-8 vector, using the *Mlu*I, *Stu*I, and *Sma*I restriction sites. **C,** Map of the construct p35SC31, comprising the full-genome cDNA clone CTV9 in the pUC35S-8 vector. Relevant *Cla*I sites to prepare ΔCla minireplicons and the external *Sfi*I sites to release the complete expression cassette are indicated. **D,** Schematic representation of the p35SΔCla9 minireplicon prepared from the full-length p35SC31 construct by a large deletion. The asterisk indicates a region with a T insertion in the construct p35SΔCla9 (nucleotide position 3,732) that was restored in the p35SΔCla295R clone (below). The AGG triplet was used to insert intron 2 of the *ST-LS1* gene shown below. The strategy and relevant *Mlu*I and *Xba*I restriction sites used for the intron insertion are outlined, with the shadowed box representing the exon or intron junctions in the new p35SΔCla295Rint viral construct (below), according to the intron sequence modified by Vancanneyt and associates (1990). Optimal consensus sequences for splicing in plants at the left and right borders are in bold (exon) or underlined (intron) characters. **E,** Detail of the T-DNA region in the BIBAC2 binary vector. RB and LB are the right and left borders. The *Not*I sites used for subcloning and the unique *Bam*HI site present in the *sacB* gene are indicated. The β-glucuronidase-neomycin phosphotransferase II and the hygromycin phosphotransferase cassettes are selectable markers encoding a β-glucuronidase-neomycin phosphotransferase II fusion peptide and hygromycin resistance, respectively. The organization and relative orientation of the minireplicon or full-length CTV cDNA in the T-DNA of the BIBAC-based constructs is shown below.

We then examined the kinetics of CTV accumulation by analyzing *N. benthamiana* leaves agroinfiltrated with plasmids BBAC31R and BBAC31Rint at 3, 6, 12, 18, and 21 dpi by enzyme-linked immunosorbent assay (ELISA), Northern blot, and real-time quantitative (q)RT-PCR. Northern blot analysis showed that accumulation of CTV gRNA and sgRNAs increased for at least 3 weeks postinfiltration (wpi) (Fig. 2D). ELISA readings also confirmed CTV increase, with positive detection beginning at 8 to 12 dpi. CTV gRNA quantification by qRT-PCR showed that, in the presence of a silencing suppressor, i) the efficiency of both plasmids was similar and ii) the viral titer increased 15 to 30 times between 1 and 3 wpi (data not shown). These results suggest the potential of BAC plasmids to efficiently establish a CTV infection in agroinfiltrated *N. benthamiana* leaves for at least three weeks, albeit viral titer at that time may differ between experiments (e.g., in six different agroinfiltration experiments with BBAC31Rint, the average number of CTV gRNA molecules per nanogram of RNAt in agroinfiltrated leaves estimated at 21 dpi ranged from $2.1 \times 10^2 \pm 3.7 \times 10^1$ to $1.1 \times 10^4 \pm 3.2 \times 10^3$).

Virion extracts semipurified from agroinfiltrated *N. benthamiana* leaves at 3 wpi were mechanically inoculated onto alemow seedlings. Infectivity obtained with BBAC31R and BBAC31Rint plasmids in three different experiments, as detected by ELISA, was similar (2:8, 0:4, and 0:4 vs. 3:9, 0:4 and 0:4 infected/inoculated citrus plants, respectively), and in all cases, the infected plants showed the symptoms characteristic of CTV-T36. CTV quantification by qRT-PCR showed that failure of mechanical transmission was usually associated with a low virus titer in agroinfiltrated *N. benthamiana* leaves (e.g., the mean virus load in *N. benthamiana* leaves agroinfiltrated with BBAC31R at harvesting time was $5.68 \times 10^3 \pm 2.4 \times 10^1$ gRNA molecules per nanogram of RNAt for the first experiment and $2.1 \times 10^2 \pm 3.7 \times 10^1$ for the second), and it was estimated that the threshold titer for successful citrus infection was around 5×10^3 to 10×10^3 gRNA copies per nanogram of RNAt, about two- to fivefold the CTV titer in infected alemow leaves.

Effect of silencing suppressors on CTV agroinfection.

Our preliminary data showed that CTV accumulation in agroinfiltrated *N. benthamiana* leaves was low, suggesting a reduced number of infection events. In an attempt to improve efficiency of agroinfection, we first compared the effect of p19 and p24, two potent silencing suppressors (Voinnet et al. 2003) encoded by *Tomato bushy stunt virus* and *Grapevine leafroll-associated virus-2* (GLRaV-2), respectively, by delivering the pCAM9R-GFP (green fluorescent protein) vector (Table 1) that comprises a version of the pCTV9R infectious clone expressing GFP (Folimonov et al. 2007; Tatineni et al. 2008; unpublished data) to *N. benthamiana* leaves, with or without a suppressor-expressing plasmid. Transient expression of pCAM9R-GFP alone resulted in a reduced number of fluorescent foci at 10 dpi that slightly increased in size at 21 dpi (Fig. 3A), suggesting inefficient CTV infection and accumulation, probably due to a plant RNA-mediated antiviral response. In contrast, the number of fluorescent foci at 10 dpi was much higher when p19 or p24 was coexpressed with the CTV vector (Fig. 3A). The highest number and size of fluorescent foci was observed at 21 dpi in leaves coinfiltrated with the p24-expressing plasmid, albeit this suppressor induced yellowing and early senescence of the infiltrated leaves.

To confirm that GFP expression was due to CTV-GFP replication, we examined the kinetics of CTV gRNA accumulation in leaves agroinfiltrated with BBAC31Rint or pCAM9R-GFP plasmids, with or without p19 or p24, along a 4-wpi time course. Coexpression of CTV with a silencing suppressor increased CTV accumulation by two- to three- (p19) or 20- to 35-fold (p24), in comparison with the cognate agroinfiltration without suppressor, as estimated by qRT-PCR (Fig. 3B) and confirmed by ELISA and Northern blot analyses (not shown). The CTV titer in leaves coinfiltrated with the pCAM9R-GFP vector was about twice that of the cognate leaves agroinfiltrated with BBAC31Rint, though the number of plasmid copies produced by pCAMBIA in bacterial cells is usually 50- to 100-fold that of the BIBAC. Coinfiltration of BBAC31Rint or pCAM9R-GFP plasmids with silencing suppressors HC-Pro (helper component proteinase) from *Tobacco etch virus* or p23 from CTV also enhanced CTV accumulation in agroinfiltrated leaves compared with the no-suppressor control, with HC-Pro and p19 inducing a similar increase in CTV titers and p23 somewhat less (not shown).

Agroinfiltration of CTV-T36 causes systemic infection of *N. benthamiana*.

The agroinoculation in infiltrated leaf areas allows infection of cells from the nucleus and does not require cell-to-cell movement, as in the normal infection process. However, our

Table 1. *Citrus tristeza virus* (CTV) constructs used in this study

Construct	Description
pCTV9	Parent pUC plasmid with full-length cDNA of the CTV-T36 genome. This clone has a frameshift at nucleotide 3,732 (Satyanarayana et al. 1999)
pCTV9R	Parent plasmid with the frameshift restored (Satyanarayana et al. 2003)
p35SC31	Parent CTV9 clone assembled into pUC35S-8 vector
p35SΔCla9	Minireplicon obtained by a large internal *Cla*I deletion in p35SC31 (Satyanarayana et al. 1999). It expresses a hybrid p23 subgenomic (sg)RNA
p35SΔCla295R	p35SΔCla9 minireplicon with the frameshift at position 3,732 restored
p35SΔCla295Rint	p35SΔCla295R minireplicon with the *ST-LS1* intron 2 sequence inserted at nucleotide position 3,652
p35SC31Rint	p35SC31 with the frameshift restored and the *ST-LS1* intron 2 sequence inserted at position 3,652
pBIN19-SfiI L3.7	pBIN19-sGFP binary vector (Chiu et al. 1996) with a *Sfi*I site added in the polylinker
pBINCTV9	pBIN19-SfiI L3.7 with the expression cassette of p35SC31 subcloned in *Sfi*I
pBINΔCla9	pBIN19-SfiI L3.7 with the expression cassette of p35SΔCla9 subcloned in *Sfi*I
pCAM9R	pCAMBIA1380 binary vector carrying an expression cassette of the CTV9R cDNA clone (Gowda et al. 2005)
pCAM9R-GFP	pCAM9R vector with the *gfp* gene (C3 version) inserted between the coat protein (CP) and the CPm open reading frames (Folimonov et al. 2007; Tatineni et al. 2008)
pCAMΔCla14R	pCAMBIA1380 carrying the expression cassette of a minireplicon similar to p35SΔCla295R (Gowda et al. 2005). It expresses the p23 sgRNA
BBAC31	BIBAC2 binary vector carrying the expression cassette of p35SC31
BBAC31R	Similar to BBAC31 but with the frameshift mutation of p35SC31 restored
BBACΔCla295R	BIBAC2 carrying the expression cassette of p35SΔCla295R
BBAC31Rint	BIBAC2 carrying the expression cassette of p35SC31Rint

time-course analyses of CTV accumulation in agroinfiltrated leaves over 4 wpi showed an increase in the number and size of fluorescent foci and in the amount of viral RNA and coat protein that suggested infection of new cells by cell-to-cell movement. Moreover, unexpectedly, we observed that, after this period, CTV-T36 was able to systemically infect upper leaves and induce symptoms in *N. benthamiana* plants, previously considered a nonhost species. Specific symptoms consisted of stunting, epinasty, and crumpled new leaves, vein clearing, and necrosis of medium and upper leaves, and plant collapse and death after 2 to 4 mpi (Fig. 4).

CTV infection in upper leaves was confirmed by ELISA, Northern blot, qRT-PCR, and fluorescence (when using a CTV-GFP vector). Repeated agroinfiltration assays using BAC- or pCAMBIA-based plasmids carrying the CTV9R sequence showed high rates of systemic infection even when no silencing suppressor was coinfiltrated with the CTV clone (Table 2), whereas no systemic infection was achieved with binary plasmids carrying the CTV9 sequence. Coexpression of a silencing suppressor was not essential for systemic infection, but it generally expedited long-distance movement and often improved infectivity, as previously observed in the agroinfiltrated leaves (Table 2A). In a first set of experiments infiltrating leaves with *A. tumefaciens* COR308, the earliest and most efficient systemic infection as detected by ELISA was achieved with p19 (73 to 93% average infected plants at 1 mpi and 83 to 100% later), followed by p23 (56 to 77% infected plants at 1 mpi and 78 to 85% later), and HC-Pro (56% infected plants at 1 mpi and 78% later), whereas no positive effect was observed with p24, even if it induced a fast accumulation of viral RNA in agroinfiltrated leaves (Table 2A). Early yellowing and necrosis of leaves agroinfiltrated with p24 likely jeopardized virus exit from these leaves, delaying long-distance movement and often reducing efficiency of systemic infection. In a second set of experiments agroinfiltrating a CTV-GFP clone with *A. tumefaciens* EHA105, the most efficient systemic infection (97% average infected plants), as detected by discontinuous fluorescence in upper leaf veins at 3 wpi, was obtained using a mix of the silencing suppressors p22 of *Tomato chlorosis virus* (ToCV) (Cañizares et al. 2008), p19, p24, and HC-Pro of *Turnip mosaic virus*, followed by individual expression of HC-Pro (69%) or p22 (59%) (Table 2B). A major improvement in the efficiency of systemic infection was obtained by subcloning the p22 silencing suppressor in the pCAMBIA vector carrying the CTV infectious clone, so that both acted always in *cis*. Agroinfiltration of leaves or injection into stems of *N. benthamiana* plants using this double vector yielded 100% systemic infection that was detected as early as 10 to 18 dpi (more than 50 plants inoculated).

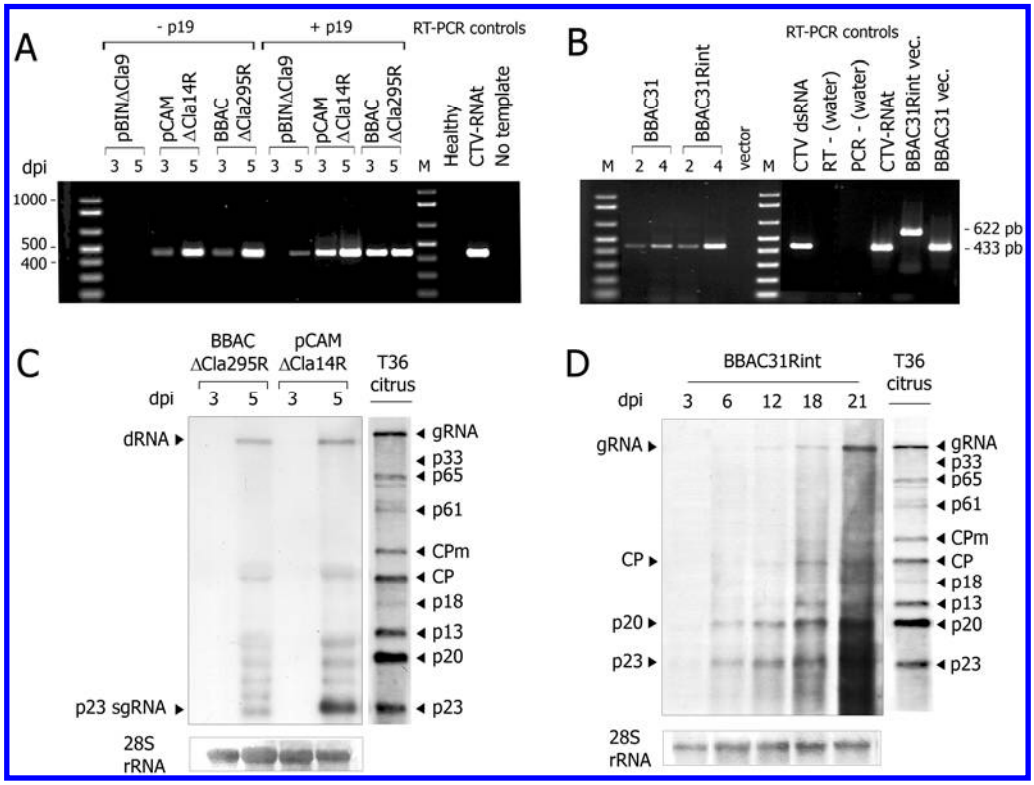

Fig. 2. Replication of the *Citrus tristeza virus* (CTV) full genome or minireplicons thereof in *Nicotiana benthamiana* leaves agroinfiltrated with binary plasmids carrying the cognate cDNA. **A,** Detection of the CTVΔCla minireplicon after agroinfiltration with plasmids pBINΔCla9, pCAMΔCla14R, or BBACΔCla295R with (+) or without (–) the p19 silencing suppressor. Reverse transcription-polymerase chain reaction (RT-PCR) amplification products obtained with primers PM118-PM119 and total RNA extracts (RNAt) from the agroinfiltrated areas at 3 and 5 days postinfiltration (dpi). Control: products amplified from healthy and CTV-infected citrus plant extracts or from distilled water. M, 1 Kb Plus DNA marker (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.), with relevant sizes of DNA fragments indicated at the left. **B,** Confirmation of intron splicing in RNA progeny derived from the construct BBAC31Rint. RT-PCR amplification products obtained from *N. benthamiana* leaves agroinfiltrated with BBAC31, BBAC31Rint, or empty BIBAC vector at 2 or 4 dpi as in A. Control: PCR amplification products from BBAC31 or BBAC31Rint plasmids and RT-PCR products obtained from CTV-infected citrus plant extracts (RNAt or dsRNA) or from distilled water. Numbers at the right indicate the size of DNA fragments amplified from the intron-containing cDNA (622 bp) and its intronless counterpart (433 bp). **C,** Northern blot analysis of RNAt (4 µg) from *N. benthamiana* leaves coinfiltrated with *Agrobacterium tumefaciens* COR308 transfected with plasmids BBACΔCla295R or pCAMΔCla14R and a vector expressing the p19 silencing suppressor at 3 or 5 dpi. The right lane shows the positions of the genomic (g)RNA and subgenomic (sg)RNAs produced by CTV-T36 in an infected citrus plant, and arrowheads in the left indicate positions of the defective RNA of the minireplicon (dRNA) and p23 subgenomic (sg)RNA. The blot was hybridized with a digoxigenin-labeled riboprobe specific for the CTV 3′ nontranslated region. Methylene blue staining of the membrane shows the 28S rRNA used as loading control. **D,** Northern blot analysis of RNAt (3 µg) from *N. benthamiana* leaves coinfiltrated with BBAC31Rint and p19 plasmids at 3, 6, 12, 18, and 21 dpi. The right lane shows the positions of the CTV-T36 gRNA and sgRNAs as in C. Hybridization and loading control also as in C.

Systemic infection resulted in high CTV accumulation in new leaves in a short period of time, generally 1 to 2 mpi (Fig. 5A). Indeed comparison of the viral titer in upper *N. benthamiana* and in citrus leaves by qRT-PCR showed that CTV accumulation was about 10- to 25-fold higher in the first than in the second host. Viral accumulation in new leaves of *N. benthamiana* was similar in plants agroinfiltrated with BBAC or with pCAMBIA plasmids, and coinfiltration with p19 often resulted in at least 1.5- to twofold increase in viral titer in comparison with plants agroinfiltrated without suppressor or coinfiltrated with p24 (Fig. 5A and B). The presence of CTV in nonagroinfiltrated new leaves could be due to systemic movement of *A. tumefaciens* (Cubero et al. 2006) followed by transient expression of CTV gRNA rather than to autonomous long-distance movement of CTV virions from agroinfiltrated leaves. We tested for the presence of *A. tumefaciens* in agroinfiltrated leaves at different times postinfiltration and in systemically infected new leaves by PCR with primers directed to the chromosomic gene *chv*E and found that i) bacterial DNA was detected in most agroinfiltrated leaf samples until 1 mpi, with occasional detection being achieved between 1 and 1.5 mpi, but no detection after this time, and ii) bacterial DNA amplification was not achieved along a time course of 2 mpi in upper leaves or even in noninfiltrated areas of the agroinfiltrated leaves showing CTV infection as detected by GFP fluorescence or by ELISA or qRT-PCR analyses, singly or together.

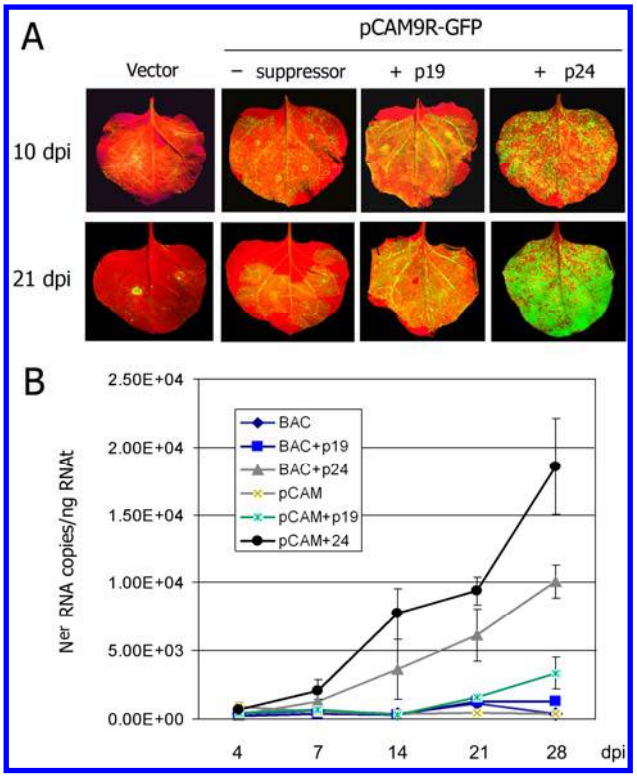

Fig. 3. Effect of silencing suppressors on *Citrus tristeza virus* (CTV) accumulation in agroinfiltrated *Nicotiana benthamiana* leaves. **A,** Fluorescence in *N. benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* COR308 harboring an empty vector or the construct pCAM9R-GFP alone or in combination with others expressing p19 or p24 silencing suppressors. Photographs taken at 10 or 21 days postinoculation (dpi), illuminating the plants with a 100 W UV hand lamp. **B,** CTV accumulation in *N. benthamiana* leaves agroinfiltrated with constructs BBAC31Rint or pCAM9R-GFP with or without silencing suppressors p19 or p24. Number of CTV genomic RNA copies per ng of total RNA extracts (RNAt) measured by quantitative reverse transcription-polymerase chain reaction (Ruiz-Ruiz et al. 2007) at 4, 7, 14, 21, and 28 dpi. Means and standard deviations were obtained from four technical replicates in two independent bioassays.

These results do not support involvement of *A. tumefaciens* in the CTV long-distance movement observed in *N. benthamiana*.

Distribution of CTV-T36 is phloem-restricted in citrus but not in *N. benthamiana*.

Monitoring CTV distribution in systemically infected *N. benthamiana* plants by tissue-print ELISA or by fluorescence emitted by CTV-GFP showed that the presence of the virus in new leaves, stems, young shoots, or roots was always associated with symptom expression (Fig. 4IIA and IIB). Plants coinfiltrated with CTV-GFP and silencing suppressors in *trans* initially (3 to 5 wpi) showed uneven virus distribution limited to the main veins or veinlets of some medium or upper leaves. Small fluorescent spots were observed during this period and the viral titer was still relatively low (Fig. 4IIC). After 1 to 2 additional weeks, the virus invaded new leaves and stems more efficiently and accumulated at higher levels, albeit it still remained phloem-limited, as revealed by tissue prints or by fluorescence observed in leaf petioles, young shoots, roots, stems, or leaves (Fig. 4ID1 and 2 and IID). At this stage CTV distribution resembled that observed in natural infections of citrus hosts (Fig. 4ID3). However, at 7 to 8 wpi, the virus was readily detected in other tissues, leading to a high viral titer (Fig. 4IE, IIE, and IIF). Systemic spread was expedited using in *cis* CTV gRNA and p22 chimeric constructs.

Virions from systemically infected *N. benthamiana* leaves (CTV-NB) observed by electron microscopy were similar to those from infected citrus tissues (Fig. 4IC). Purified virion preparations from these new leaves were infectious on alemow plants upon mechanical inoculation (Table 3), and reproduced the symptoms characteristic of the wild CTV-T36 (leaf cupping, vein clearing, and stunting). The ratio of infected plants obtained by inoculation with CTV-NB preparations from systemically infected *N. benthamiana* plants agroinfiltrated with BAC- or pCAMBIA-based plasmids were similar and usually averaged between 73 and 87%. Moreover, the high viral titer of systemically infected *N. benthamiana* leaves enabled us to infect citrus using crude sap extracts, albeit transmission with this inoculum source was less efficient and symptom onset was delayed (29 to 33% average infected citrus plants at 2 mpi) (Table 3). Distribution of CTV or CTV-GFP in citrus slash-inoculated with CTV-NB virions was restricted to the phloem of young shoots, leaf veins or petioles, stem bark or roots, as in naturally infected citrus, and the virion morphology observed at the electron microscope was also normal (Supplementary Fig. 1). These results confirm that CTV passage through *N. benthamiana*, in which the virus is not phloem-limited, does not change its original biological characteristics.

Summarizing, agroinfection of the presumed nonhost species *N. benthamiana* using BAC- or pCAMBIA-based plasmids to incite a systemic infection provides a novel genetic system for CTV that is simpler, faster, and more efficient than the available protoplast-based system (Satyanarayana et al. 1999, 2001).

DISCUSSION

Previous efforts to agroinfect citrus with CTV using binary plasmids and different silencing suppressors, oncogenic or disarmed *A. tumefaciens* strains, and agroinfiltration conditions were unsuccessful (our unpublished data). CTV could not be detected in agroinfiltrated tissues by ELISA, conventional RT-PCR, or fluorescence (when using CTV-GFP). Occasionally, a faint amplification was detected by qRT-PCR that later disappeared, suggesting a subliminal infection that did not progress. For this reason, and because CTV-T36 was known to replicate in *N. benthamiana* protoplasts (Navas-Castillo et al. 1997; Satyanarayana et al. 1999), we tried to develop a genetic sys-

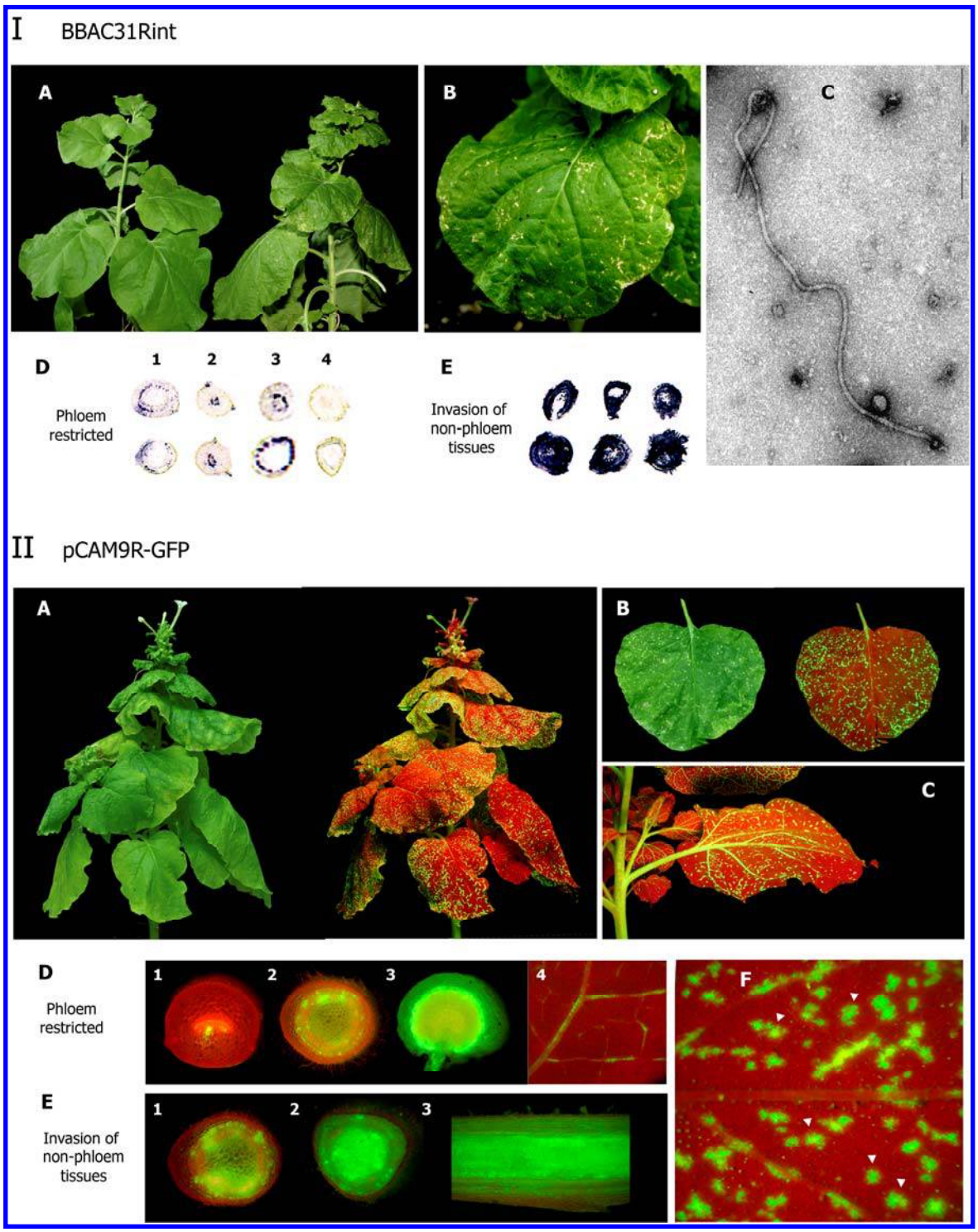

Fig. 4. Systemic spread and distribution of *Citrus tristeza virus* (CTV) in *Nicotiana benthamiana* after agroinfiltration with **Panel I,** constructs BBAC31Rint or **Panel II,** pCAM9R-GFP. **I, A** and **B,** Symptom expression and **D** and **E,** CTV distribution in a *N. benthamiana* plant systemically infected. **A,** Infected plant (right) showing leaf epinasty and vein chlorosis in comparison with a healthy control (left). **B,** Medium leaf showing vein necrosis. **C,** Negatively-stained, gradient-purified CTV virion from a systemically infected *N. benthamiana* plant (the bar indicates 500 nm). **D,** Tissue-print immunoblots from systemically infected *N. benthamiana* stem (1) and upper leaf petiole (2) at 1 month postinoculation (mpi), CTV-infected citrus leaf petiole (3) or healthy *N. benthamiana* leaf petiole (4). Purple-stained foci indicate the presence of CTV coat protein (CP) that, at this stage, is phloem-restricted. **E,** Similar tissue-print immunoblots from systemically infected *N. benthamiana* leaf petioles or stems at 2 mpi. General purple staining indicates that CTV has invaded nonphloem tissues. **II, A** and **B,** Symptom expression and **A** through **F,** CTV distribution in *N. benthamiana* plants infected with a green flourescent protein (GFP)-expressing CTV virus. **A,** Systemically infected *N. benthamiana* plant at 1 mpi, showing leaf epinasty and vein chlorosis under day light (left) or GFP fluorescence under UV light (right). **B,** Spatial association between vein chlorosis (left) and the presence of GFP fluorescence (right). **C,** Progress of CTV infection along stem, leaf petiole, and veins. **D,** CTV-GFP distribution in phloem tissue of young petiole (1), shoot (2), root (3), or leaf veins (4) in systemically infected *N. benthamiana* at 1 mpi. **E,** Distribution in shoots (1 and 2) and roots (3) at 2 mpi, showing the presence of the virus in nonphloem tissues. **F,** Distribution in leaves at 2 mpi, showing CTV accumulation in main veins and veinlets but also in mesophyll cells (arrowheads).

tem based on *N. benthamiana* plants as intermediate host to produce virions by monitoring the kinetics of CTV accumulation in agroinfiltrated leaves. Former experiments with this system showed that mechanical inoculation of citrus plants with virion extracts from infiltrated leaves at 7 to 8 dpi was inefficient, likely due to low virus titers (Gowda et al. 2005). Here, we observed that CTV increase in these leaves continued until at least 3 wpi and that the size and number of fluorescent foci in leaves agroinfiltrated with CTV-GFP also increased, suggesting that CTV not only replicated in *N. benthamiana* cells but it was able to move cell-to-cell. Yet, the movement into nonvascular cells was much more dramatic in the upper, systemically infected tissues. Movement of closteroviruses is known to occur after virion assembly (Alzhanova et al. 2001; Dolja 2003; Esau et al. 1967; Peremyslov et al. 2004), a process that requires the cooperation of at least four viral proteins (Alzhanova et al. 2007; Napuli et al. 2003; Peremyslov et al. 2004; Satyanarayana et al. 2000). Additionally, the leader proteinases (Liu et al. 2009; Peng et al. 2001, 2002), the HSP70 homolog (Medina et al. 1999; Prokhnevsky et al. 2005), and the small transmembrane protein p6 (Alzhanova et al. 2000; Peremyslov et al. 2004; Tatineni et al. 2008) are required for cell-to-cell movement. Our finding that CTV-T36 can move cell-to-cell indicates that the above viral proteins properly interact with *N. benthamiana* factors involved in this process. Whether other CTV genotypes genetically distant from CTV-T36 (Hilf et al. 2005; Martín et al. 2009; Moreno et al. 2008) are also capable of replication and movement in *N. benthamiana* plants remains to be proved.

Occurrence of systemic infection and symptoms in agroinfiltrated plants at 4 wpi or later was even more surprising. Systemic infection of *N. benthamiana* by agroinoculation of *Beet*

Table 2. Efficiency of systemic infection in *Nicotiana benthamiana* plants agroinfiltrated with a full-genome *Citrus tristeza virus* (CTV) cDNA clone using bacterial artificial chromosomes (BAC) or pCAMBIA plasmids and different silencing suppressor proteins

	Infectivity[a]				
Clone	**None**	**p19[b]**	**p24[c]**	**HC-Pro[d]**	**p23[e]**
Section A					
BBAC31Rint	4/6(5/6)	3/3	1/5	3/4(4/4)	3/4(4/4)
	4/5(5/5)	3/3	1/4(2/4)	2/4(3/4)	2/3(3/3)
	5/8(6/8)	7/8(8/8)			3/3
	2/4(3/4)	4/4	1/4	2/5(3/5)	2/3(3/3)
	1/5(2/5)	5/5	2/5(4/5)	2/5(3/5)	2/5(3/5)
	3/5	4/5(5/5)	2/5	4/5(5/5)	1/5(2/5)
Mean (%)	57.6(72.7)	92.9(100)	30.4(43.5)	56.5(78.3)	56.5(78.3)
BBAC31R	5/6(6/6)	2/3			3/4(4/4)
	1/4(2/4)	2/4(4/4)			1/3
	2/3(3/3)	3/3			3/3
	2/5(3/5)	3/3			3/3
	1/4	4/4			
Mean (%)	50(68.2)	82.4(94.1)			76.9(84.6)
pCAM9R	0/4	4/5(5/5)	1/5(3/5)		
	3/5(4/5)	5/5	4/5		
	3/5(4/5)	4/5(5/5)	1/5(3/5)		
	1/5(3/5)	4/5			
	4/5(5/5)	5/5	3/5		
	0/5(2/5)				
Mean (%)	37.9(62.1)	88(96)	45(65)		
pCAM9R-GFP	0/4	6/7(7/7)	4/7		
	0/5	3/5	0/5(1/5)		
	0/3	3/4	1/4(3/4)		
	1/3	2/3(3/3)	1/3(2/3)		
	1/7(5/7)	4/6(5/6)	1/7(6/7)		
	2/5	4/5	1/5		
Mean (%)	14.8(29.6)	73.3(83.3)	25.8(54.8)		
	p22[f]	**p19[b]**	**p24[c]**	**HC-Pro[g]**	**p19+p22+p24+HC-Pro[h]**
Section B					
pCAM9R-GFP	10/14				
	7/8	4/8	3/8	4/8	4/4
					10/10
	5/8	4/8	1/8	5/8	7/8
	3/8	1/8	5/8	6/8	7/9
	2/8	1/8	3/8	7/8	8/8
					48/48
					8/8
					6/6
Mean (%)	58.7	31.2	37.5	68.7	97

[a] Expressed as the number of systemically infected plants to the number of agroinfiltrated plants and as the mean percentage of infected plants. Each row corresponds to a different experiment. In section A, infection in upper leaves was detected by enzyme-linked immunosorbent assay or fluorescence expression (CTV-GFP clones) at 4 to 6 weeks postinoculation (wpi) or at 8 wpi or later (within brackets). In section B, systemic infection was detected by discontinuous fluorescence in leaf veins at 3 wpi.

[b] Coinfiltration with a vector expressing the p19 suppressor protein of *Tomato bushy stunt virus*.

[c] Coinfiltration with a vector expressing the p24 protein of GLRaV2.

[d] Coinfiltration with a vector expressing HC-Pro of *Tobacco etch virus*.

[e] Coinfiltration with a vector expressing the p23 protein of CTV.

[f] Coinfiltration with a vector expressing the p22 protein of *Tomato chlorosis virus*.

[g] Coinfiltration with a vector expressing HC-Pro of *Turnip mosaic virus*.

[h] Coinfiltration with a vector expressing a mix of cultures expressing b, d, f, and g suppressors.

yellows virus (BYV), *Lettuce infectious yellows* (LIYV), and GLRaV-2, also members of the family *Closteroviridae*, has been reported (Chiba et al. 2006; Liu et al. 2009; Prokhnevsky et al. 2002; Wang et al. 2009); however, while this species was known as an experimental systemic host for these viruses, it was considered a nonhost for CTV. Failure to infect *N. benthamiana* by mechanical inoculation with virion extracts may be related with the small number of viable particles reaching the cytoplasm and gRNA silencing before expression of the CTV silencing suppressors. Stunting, epinasty, and vein clearing and necrosis caused by CTV in systemically infected *N. benthamiana* plants resemble those incited in Mexican lime, which are diagnostic for CTV; however, crumple of young leaves is not observed in citrus, and seedling yellows, stem pitting, and tristeza decline symptoms, characteristic of different citrus species or scion-rootstock combinations (Moreno et al. 2008), cannot be observed in *N. benthamiana*. These findings suggest that, while some interactions between CTV and host factors inciting symptoms might be similar in citrus and *N. benthamiana*, others must be host-specific, in agreement with previous observations that ectopically expressed p23 incites CTV-like symptoms only in citrus plants (Fagoaga et al. 2005).

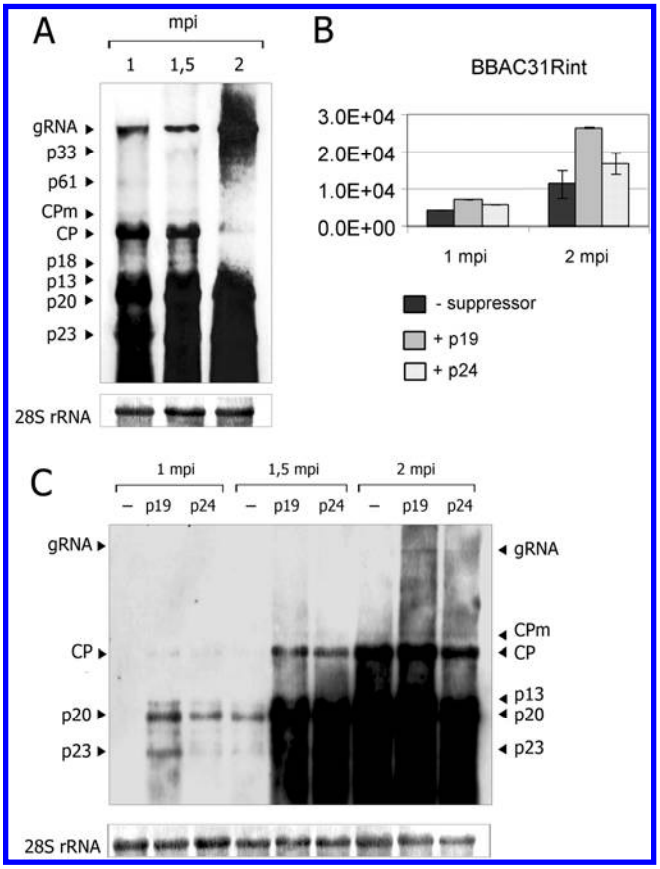

Fig. 5. *Citrus tristeza virus* (CTV) accumulation in new leaves of systemically infected *Nicotiana benthamiana* plants agroinfiltrated with or without silencing suppressors. **A,** Northern blot analysis of total RNA extracts (RNAt) (3 µg) extracted at 1, 1.5, and 2 months postinoculation (mpi) from the upper leaves of *N. benthamiana* coinfiltrated with the construct CTV-BBAC31Rint and the silencing suppressor p19. Positions of the genomic (g)RNA and relevant subgenomic (sg)RNAs are indicated by arrowheads. The membrane was hybridized with a digoxigenin-labeled riboprobe specific for the 3′ terminal region of the CTV gRNA. Methylene blue staining of the membrane shows the 28S rRNA used as loading control. **B,** Number of CTV gRNA copies per nanogram of RNAt from new leaves of systemically infected *N. benthamiana* plants at 1 or 2 mpi, measured by quantitative reverse transcription-polymerase chain reaction (Ruiz-Ruiz et al. 2007). Means and standard deviations were obtained from four technical replicates in two different bioassays. **C,** Northern blot analysis of RNAt (3 µg) extracted from new leaves of systemically infected *N. benthamiana* at 1, 1.5, and 2 mpi, with the construct pCAM9R alone or with silencing suppressors p19 or p24. Other details as outlined in A.

Since *A. tumefaciens* can systemically spread in several host species (Cubero et al. 2006), the presence of CTV in upper *N. benthamiana* leaves could be due to movement of the bacterium followed by local expression of CTV; however, failure to PCR-amplify a bacterial sequence out of the infiltrated leaf area does not support this hypothesis. It seems more likely that the potential long-distance movement factors of CTV-T36 are capable of proper interactions with *N. benthamiana* factors to allow systemic infection and that this would account for most, if not all, CTV accumulation in the upper leaves.

Systemic infection was obtained using a high or a low copy–number vector to deliver the CTV genome, and with or without coinfiltration with a vector expressing a silencing suppressor, albeit the ratio of systemically infected plants and the time elapsed between agroinfiltration and virus detection in the upper leaves was variable among experiments. The main advantage of using a low copy–number vector like BIBAC2 and an intron inserted in the CTV cDNA is that it can be easily manipulated in the lab without the bacterial toxicity and plasmid reorganization problems often found with CTV cDNA cloned in high copy–number vectors. Also, although the number of primary infections is greatly increased in plants coinfiltrated with a silencing suppressor, as previously observed with BYV (Chiba et al. 2006), the efficiency of systemic infection seems less affected by this factor, in agreement with results obtained with LIYV (Wang et al. 2009). A high ratio of systemically infected plants (almost 100%) was achieved in about three weeks, coinfiltrating plants with a mix of four silencing suppressors, a period that was reduced to less than 2 wpi when the p22 suppressor of the crinivirus ToCV (Cañizares et al. 2008) was expressed in *cis* in the nucleus of the plant cells by cloning it in the CTV-expressing vector. To our knowledge, this is the first time that expression of a silencing suppressor in *cis* has been used to improve agroinoculation of a plant virus. These results provide a genetic system for CTV that is easier, faster, and more reliable than protoplast transfection with RNA

Table 3. Infectivity of *Citrus tristeza virus* (CTV) virions obtained from systemically infected *Nicotiana benthamiana* on mechanically inoculated alemow plants

Inoculum source	Virions[a]	Crude sap[b]
Upper leaves from *N. benthamiana* agroinfiltrated with		
BBAC31Rint clone	2/3	1/4
	3/4	2/7(3/7)
	3/4(4/4)	3/20(4/20)
	4/4	1/4(2/4)
Mean (%)	80(86.7)	20(28.6)
BBAC31R clone	2/4	1/5
	1/3	1/4(2/4)
	3/4(4/4)	
	4/4	
Mean (%)	66.7(73.3)	22.2(33.3)
pCAM9R clone	4/4	
	3/4	
	4/5	
Mean (%)	84.6	
Control[c]	3/3	
	4/4	
Mean (%)	100	

[a] Gradient purified virions. Infectivity expressed as the number of infected citrus plants to the number of inoculated plants at 1 to 1.5 months postinoculation (mpi) and as the mean percentage of infected plants. Numbers within brackets indicate infectivity at 3 mpi or later.
[b] Infectivity at 2 or ≥3 mpi (within brackets).
[c] Citrus bark infected with CTV 947R

transcripts (Satyanarayana et al. 1999, 2001). Agroinfiltration of *N. benthamiana* plants does not require preparation of RNA transcripts and protoplasts, avoids loss of experiments due to protoplast contamination, and reduces to one fourth or less the time necessary to obtain enough CTV virions to mechanically inoculate citrus plants.

Systemic infection of *N. benthamiana* plants was critical for the last step of the genetic system, i.e., efficient infection of citrus plants by slash-inoculation with virion extracts. Early experiments using virion extracts from agroinfiltrated leaves at 7 to 8 dpi (Gowda et al. 2005) were usually unsuccessful, due to low virus accumulation during this period. Indeed time-course accumulation measures showed a 15- to 30-fold increase of CTV titers in agroinfiltrated leaves between 1 and 3 wpi, sometimes reaching the threshold titer for successful transmission to citrus (about 5×10^3 to 10×10^3 gRNA copies per nanogram of RNAt). Virion accumulation in the upper leaves was higher, particularly after 7 wpi, when CTV invaded not only the phloem but other parenchyma tissues as well. This high virion concentration enabled easy transmission to citrus, even using crude leaf extracts. Contrasting with *N. benthamiana*, citrus plants infected with the same virions showed a normal CTV distribution strictly restricted to the phloem, indicating that passage through *N. benthamiana* did not alter movement characteristics of CTV in citrus. This distribution is likely related to the strong silencing reaction incited by CTV infection in citrus (Fagoaga et al. 2006), and it can be partially counteracted by ectopic expression of the p23 silencing suppressor in transgenic plants (Fagoaga et al. 2011). Unrestricted spread of CTV in *N. benthamiana* in advanced stages of the infection suggests a weaker silencing reaction from this host in comparison with citrus, perhaps due to the lack of an active salicylic acid- and virus-inducible RNA-dependent RNA polymerase (*NbRdRP1m*) involved in silencing of some viruses that makes *N. benthamiana* very susceptible to those viruses (Yang et al. 2004).

In summary, agroinfiltration of CTV-T36 to *N. benthamiana* plants provided the first herbaceous experimental host for this closterovirus that, in nature, is restricted to citrus. Although CTV was aphid transmitted to *Passiflora gracilis* and *P. caerulea* (Müller et al. 1974; Roistacher and Bar-Joseph 1987), these are perennial vines that offer no experimental advantage compared to citrus. Availability of a well known herbaceous systemic host for which molecular information is rapidly increasing will be a most useful tool to start dissecting CTV-host interactions, particularly those related to within-plant movement and symptom expression. The high virus accumulation in systemically infected leaves makes it easy to assay new mutants in citrus within a workable time.

MATERIALS AND METHODS

Virus source and plant growth.

The isolate 947R is a clonal virus population obtained from a cDNA clone of the CTV genotype T36 from Florida that has biological characteristics indistinguishable from the wild CTV-T36 (Satyanarayana et al. 2001). Isolate 947R is maintained in Mexican lime and alemow seedlings in a contained, temperature-controlled (18°C night and 26ºC day) greenhouse. Citrus plants were grown in an artificial potting mix (50% peat moss, 50% sand) and were fertilized by a standard procedure. *N. benthamiana* plants were grown in a customized growth chamber kept at 23 to 24ºC constant temperature and 50 to 60% relative humidity with a 16-h light and 8-h dark photoperiod.

RNA extraction.

Extracts enriched in CTV dsRNA were obtained from 5 to 10 g of citrus bark as reported previously (Moreno et al. 1990). RNAt were prepared from 1 to 3 g of young citrus bark or from 100 to 300 mg of *N. benthamiana* leaf by grinding tissue to powder with liquid nitrogen and, then, using a standard phenol-chloroform-isoamyl alcohol extraction protocol (Ruiz-Ruiz et al. 2007) or the RNeasy plant mini kit (Qiagen, GmbH Qiagen, Hilden, Germany). When needed, RNA was further purified with the RNeasy cleanup kit (Qiagen) and was resuspended in 25 µl of RNase-free water, and in all cases, was treated with RNase-free DNase (Turbo DNA-free, Ambion, Inc., Applied Biosystems, Austin, TX, U.S.A.) before using it as template for RT-PCR or real-time qRT-PCR amplification assays.

Plasmid constructs, binary plasmids and bacterial strains.

A pUC119 vector was initially modified by site-directed mutagenesis to introduce a set of suitable restriction sites, two external *Sfi*I sites flanking a *Stu*I-*Mlu*I-*Sma*I minilinker. Plasmid pUC35S-8 (Fig. 1B) was generated from this modified pUC119 after three subcloning steps: i) a double-enhanced 35S promoter (35S × 2) of the CaMV was PCR-amplified from a pKYLK plasmid (plasmid p35Se-NOS-B provided by J. J. López-Moya, C.S.I.C., Barcelona, Spain) and was ligated as a *Pst*I-blunt ended fragment after *Pst*I-*Stu*I digestion of the plasmid, ii) the NOS-t from the plasmid pBI101 was excised and cloned as a *Sac*I-*Eco*RI fragment, and iii) the antigenomic ribozyme sequence of the *Hepatitis delta virus* was PCR-amplified from vector 2.0 (provided by A. Ball, University of Alabama, Birmingham, U.S.A.), and then, was cloned as a blunt end *Sac*I fragment into the *Sma*I-*Sac*I–digested plasmid.

The full-genome CTV cDNA clone p35SC31 was assembled into the new pUC35S-8 vector by three successive cloning steps. Two cDNA fragments (Fig. 1A, fragments A and B) were PCR-amplified, using appropriate primers based on the T36 nucleotide sequence (U16304) and the pCTV9 infectious clone as template (Satyanarayana et al. 1999, 2001). The two fragments were successively cloned in pUC35S-8; fragment A spanning the 5′ terminal 3,703 nt was *Mlu*I-digested and was inserted into the *Stu*I-*Mlu*I digested pUC35S-8 vector, and then, fragment B spanning nucleotides 14,773 to 19,296 was *Mlu*I-digested and was inserted into the *Sma*I-*Mlu*I–digested vector containing fragment A. Finally, an internal *Mlu*I fragment derived from pCTV9 plasmid (Fig. 1A, fragment C) was cloned into the appropriately digested vector to engineer the full-length clone. Minireplicon p35SΔCla9 was generated from the full-genome clone p35SC31 by a large *Cla*I internal deletion, as previously described (Satyanarayana et al. 1999). A version of this replicon with the frameshift at position 3,732 properly restored was synthesized as reported by Satyanarayana and associates (2003) to obtain the vector p35SΔCla295R (Fig. 1D).

To reduce bacterial toxicity of the restored CTV sequence, an intron was inserted in the toxic region (Satyanarayana et al. 2003) to disrupt ORF 1a. The intron 2 sequence of the *ST-LS1* gene from potato (Eckes et al. 1986) was PCR-amplified from the p35S-GUSintron plasmid (Vancanneyt et al. 1990), using a set of primers designed to optimize the consensus sequences for splicing in plants. The intron sequence was introduced at nucleotide positions 3,652 or 3,744 in the CTV genome, using an overlap-extension PCR technique (Ho et al. 1989) (Fig. 1D). The amplified fragment was digested and cloned between unique restriction sites *Mlu*I and *Xba*I, present in p35SΔCla295R, to obtain the construct p35SΔCla295Rint. A *Sma*I-*Not*I cDNA fragment obtained after digestion of the clone p35SC31 was ligated into the similarly digested p35SΔCla295Rint vector to obtain the full-length construct p35SC31Rint. The CTV full-length cDNA clones pCTV9, pCTV9R, and those derived from them were grown in *E. coli*

JM109 as described previously (Satyanarayana et al. 1999, 2001). The size of the *E. coli* colonies was measured with a stereoscopic microscope at 1 and 2 days after plating.

Binary plasmids pBIN19, pCAMBIA1380, and BIBAC2 were utilized for agroinoculation experiments. Clones pBINCTV9 and pBINΔCla9 were obtained after *Sfi*I-digestion of p35SC31 and p35SΔCla9 plasmids, respectively, followed by ligation into the *Sfi*I-restricted vector pBIN19-SfiI L3.7, a modified version of pBIN19-sGFP (Chiu et al. 1996) obtained by addition of a *Sfi*I site. Constructs pCAMΔCla14R and pCAM9R, based on the pCAMBIA1380 plasmid, have been described previously (Gowda et al. 2005). The gene for GFP expression (GFP-C3 version) was inserted into the sequence of the CTV9R cDNA, between the CP and CPm ORF under the BYV CP controller element (Folimonov et al. 2007; Tatineni et al. 2008), in order to obtain the pCAM9R-GFP vector. The BIBAC2 vector (22.5 kb) replicates in *E. coli* and *A. tumefaciens* cells, and it contains two *Not*I sites flanking the unique *Bam*HI cloning site and selectable markers for resistance to kanamycin (neomycin phosphotransferase II) and hygromicin (hygromycin phosphotransferase) (Hamilton 1997) (Fig. 1E). Unless otherwise indicated, CTV expression cassettes were excised from the plasmid with *Sfi*I, were ligated to synthetic *Sfi*I linkers containing an internal *Not*I site, and were then digested with the latter enzyme. The CTV *Not*I-restricted cassettes were then ligated to purified *Not*I-restricted BIBAC2 plasmid, and the ligation mix was used to transform *E. coli* DH10B competent cells by electroporation with a Gene Pulser (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). Transformed cells were selected on LB plates containing kanamycin (80 mg/liter) and sucrose (5 to 10%). About 20 to 50 ng of plasmid, purified by standard BAC-miniprep, were used to transform the *A. tumefaciens* COR308 strain (UIA143 pMP90 pCH32; Hamilton 1997) by electroporation. The helper plasmid pCH32 carries additional copies of *virG*, *virE1*, and *virE2* genes from *A. tumefaciens*, and it is selected on LB medium containing 10 mg of tetracycline per liter. *A. tumefaciens* EHA105 was also used in some experiments. Transfected cells were selected on LB medium containing kanamycin (50 mg/liter) and nalidixic acid (20 mg/liter). Occasionally, some oncogenic *A. tumefaciens* strains were used, selecting them with appropriate antibiotics. Additional details on the constructs can be supplied by the authors upon request.

Agroinoculation and CTV detection.

In general, colonies of *A. tumefaciens* (strain COR 308) transfected with BBAC, pBIN, or pCAMBIA plasmids carrying the full-length CTV cDNA were grown overnight at 28ºC in LB medium supplemented with 50 mg of kanamycin per liter, 5 mg of tetracycline per liter, 10 mM MES (morpholineethanesulfonic acid), and 20 μM acetosyringone. Bacteria were collected by centrifugation at 6,000 × *g* for 15 min at room temperature, were resuspended in LB medium supplemented with 10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 μM acetosyringone, and the concentration was adjusted to an optical density at 600 nm value of 1. After 3 to 5 h of induction in this medium, bacterial suspensions were infiltrated on fully expanded *N. benthamiana* or citrus leaves using a syringe. In coinfiltration experiments, *A. tumefaciens* cultures transfected with CTV-carrying binary plasmids (or empty plasmids) and others transfected with plasmids expressing silencing suppressor proteins were mixed in a 2:1 ratio (CTV clone/silencing suppressor) prior to infiltration.

CTV infection in *N. benthamiana* was monitored in the infiltrated leaves by double-antibody sandwich-ELISA using a mix of monoclonal antibodies 3DF1 and 3CA5 (Vela et al. 1986), by RT-PCR techniques, Northern blot analysis, or fluorescence observation (when CTV-GFP was used) once a week during the first month after inoculation, and then, every one or two weeks in the upper leaves. GFP fluorescence was generally observed with a long-wavelength UV light hand-lamp (Black Ray model B100AP, UV products, Upland, CA, U.S.A.) and was photographed using a Canon G7 digital camera combined with a yellow filter. In each experiment, Northern blot and RT-PCR detection and quantification (discussed below) were performed on a pool of leaf fragments from all plants of the same treatment. Data provided are from at least two independent experiments with at least two replicates. Images of fluorescence distribution in infected tissues were taken with a Leica stereoscope (Leica, Heidelberg, Germany) provided with UV illumination and a GFP filter. Immunosorbent electron microscopy was performed as described by Milne (1993), using the monoclonal antibodies 3DF1 and 3CA5, and the grids were examined in a Philips 100 CM10 electron microscope at 80 kV.

Northern blot analysis.

For Northern blot analysis of CTV RNA, RNAt aliquots (3 to 5 μg) were separated by 1% formaldehyde agarose gel electrophoresis in MOPS (morpholinepropanesulfonic acid) buffer, were electroblotted onto positively charged nylon membranes (Roche Diagnostics, F. Hoffmann-La Roche AG, Basel, Switzerland), and were hybridized with a digoxigenin-labeled riboprobe specific for the 3′ terminal region of the CTV genome (Satyanarayana et al. 1999).

RT-PCR and qRT-PCR.

Correct splicing of the intron inserted in the CTV cDNA after agroinoculation and transcription in the plant nucleus was checked by RT-PCR, using primers PM118 (5′-GTGTTATCATGCATCGGAGGCG-3′) and PM119 (5′-ACCGGGGAATTTTGATTCTAATC-3′) directed to regions flanking the intron insertion point in the CTV gRNA (positions 3,652 and 3,744 in the CTV-T36 sequence). RT was performed using these primers, 1 μg of DNase-treated RNAt and SuperScript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.), in the conditions reported (Ruiz-Ruiz et al. 2006). A control reaction without reverse transcriptase was carried out for each sample. PCR amplification was performed in a 50-μl reaction mix containing a 1-μl aliquot of the RT reaction, primers PM118 and PM119, and *Taq* DNA polymerase (Roche Diagnostics) (Ruiz-Ruiz et al. 2006). Negative controls included reactions using water instead of RNAt or RNAt extracts from healthy plants or from plants agroinfiltrated with an empty binary vector, whereas similar reactions with RNAt or dsRNA-rich extracts from CTV-infected citrus plants or plasmid DNA containing CTV cDNA with or without the intron were used as positive control. PCR amplification with primers PM118 and PM119 is expected to yield DNA fragments of 622 bp with the intron-containing template or 433 bp with the intronless template. PCR products were purified using the GeneClean kit (QBiogene, Inc, Carlsbad, CA, U.S.A.) and were sequenced in both directions to confirm the correct RNA splicing in planta.

Quantitative assays (qRT-PCR) were performed in a LightCycler platform (Roche Diagnostics) with 20-μl glass capillaries in the conditions reported (Ruiz-Ruiz et al. 2007). In each run, control samples included RNAt from healthy citrus or *N. benthamiana*, water instead of RNAt extract, and at least one RNA transcript dilution of the standard curve. Also, for each plant extract, real-time RT-PCR with and without reverse transcriptase were run in parallel to ensure the absence of binary plasmid DNA or other contaminant DNA template. The absolute number of T36 gRNA copies per nanogram of RNAt was estimated using an external standard curve prepared with RNA

transcripts including the amplified region, as described previously (Ruiz-Ruiz et al. 2007).

Indexing in indicator plants.

The infectivity of CTV virions generated in agroinfiltrated *N. benthamiana* leaves or in plants systemically infected was tested by slash-inoculation of four or five alemow plants (Garnsey et al. 1977) using gradient purified extracts (Satyanarayana et al. 2001) or crude sap extracts as indicated. A similar number of indicator plants was inoculated with virion extracts from citrus plants infected with the 947R isolate of CTV-T36 as control. CTV infection in the new leaves was detected at 1 to 2 mpi by ELISA.

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