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# Efficient Infection of Citrus Plants with Different Cloned Constructs of *Citrus tristeza virus* Amplified in *Nicotiana benthamiana* Protoplasts

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ABSTRACT. Infection of citrus is a critical but difficult step for the evaluation of cloned cDNA constructs of *Citrus tristeza virus* (CTV). So far, all attempts to directly infect citrus using either *in vitro* transcripts of CTV cDNA constructs or virus progeny extracted from transcript-inoculated *Nicotiana benthamiana* protoplasts have not been successful. We observed that RNA transcripts infect only 0.1% or less of *N. benthamiana* protoplasts, while virions can infect 80% or more of individual protoplasts. A multi-step procedure was devised to amplify the initial low-level infection in protoplasts to obtain quantities of virus sufficient for infection of citrus plants. Virions were extracted from transcript-inoculated *N. benthamiana* protoplasts and used for 4 to 10 sequential protoplast passages to maximize virus titer. Increased amplification levels were monitored by Northern blot analysis and ELISA. Extracts from the final protoplast passages were further concentrated by centrifuging through sucrose cushions, and the virus-containing zones were harvested and used for stem inoculation of citrus plants. We have successfully infected citrus plants with 38 constructs of CTV with this procedure, and plant infection rates frequently approach 100%.

*Index words*. Protoplasts, virions, amplification, concentration, stem inoculation.

*Citrus tristeza virus* (CTV) is a member of the Closteroviridae and the largest plant virus. CTV is an extremely complex RNA virus, with a genome approximately 20 kilobases (kb) in size containing 12 ORFs encoding at least 19 proteins (8, 11). CTV is phloem-limited and transmitted semi-persistently by aphids and also by vegetative propagation. It poses a serious economic threat to citrus production worldwide. Isolates range from mild to severe, with a wide variety of responses elicited in different host/rootstock combinations. Stem pitting in sweet orange and grapefruit results in a loss of tree vigor and reduced fruit size and yields. Tristeza decline, which kills trees grafted on sour orange rootstocks, has essentially negated the use of sour orange in Florida and other citrus growing areas where CTV is endemic. It has been difficult to find decline resistant rootstocks that also have the desirable horticultural characteristics of sour orange and its tolerance to foot rot, blight, and other diseases of citrus. Intensive research has been done to improve controls for stem pitting and decline diseases of citrus. Although CTV is a difficult virus to study, extensive progress has been made on its molecular characterization. The complete viral sequence was determined (8, 11) and a full-length infectious cDNA clone was created from this sequence (12). Development of a method to infect protoplasts of *Nicotiana benthamiana* with transcripts from the infectious clone (10, 12) allowed the biology of various mutants of CTV to be studied. While *N. benthamiana* protoplasts were highly susceptible to infection by intact virus, only a small percentage (0.1% or less) were infected by transcripts. All attempts to directly infect citrus plants with RNA transcripts from cloned cDNA or with cDNA failed. Effective virus amplification

had to be accomplished through the use of serial passaging of protoplasts to produce inoculum to establish a few infections in citrus plants to confirm Koch's postulates (13). While this demonstrated infection of citrus with cloned constructs of CTV, the method used was unreliable and inefficient. Infections with seven constructs were established in citrus plants with only an 8% efficiency (17 infected plants in 220 total attempts, unpublished). This low and inconsistent level of plant infection was a major obstacle to engineering constructs to identify how induction of diseases and other properties are regulated. So, we began to look at ways to obtain more efficient infection.

Previous work with CTV extracted from infected citrus plants showed that plants could be infected with intact virions via mechanical means, such as stem slashing and bark flap inoculation, which introduced CTV into plants by phloem wounding (2). Infections at low levels could be obtained using extracts prepared by dicing young bark of infected plants, which released intact virions from the tissue. Infection rates were substantially increased by concentrating the virus in these extracts on sucrose step gradients or sucrose cushions (3, 4). Selection of appropriate receptor host plants for inoculation was also important (9). Varieties such as Alemow, Mexican lime, and Etrog were more susceptible than other hosts, and infections could frequently be detected by presence of vein clearing and leaf cupping symptoms.

These studies with infectious extracts indicated the approximate titer of CTV that would be needed to effectively establish infection in susceptible citrus hosts. Using this information, we developed and evaluated a method that combined optimal replication of CTV mutants in *N. benthamiana* protoplasts with production of sufficient extract from protoplasts to prepare inoculum concentrated through sucrose cushions. The method and its successful application with multiple cloned constructs of CTV are presented.

## MATERIALS AND METHODS

**Preparation and inoculation of *Nicotiana benthamiana* mesophyll protoplasts.** *N. benthamiana* plants were grown at 27°C with a 14 h photoperiod under 400 watt metal halide lamps in sterilized Scotts Metromix 500 soil and fertilized on a weekly basis. The newest fully expanded leaves were collected from plants that were 6 to 7-weeks-old and surface sterilized. Leaves were slashed at 1 mm intervals on the bottom side, placed in an enzyme solution containing 1.0% Cellulase Onozuka RS (Yakult Pharmaceutical, Japan) and 0.5% Macerase pectinase (Calbiochem, La Jolla, CA, USA), and incubated at 27°C for 18 to 20 h in the dark. The protoplasts were then prepared as described by Navas-Castillo et al. (10). After isolation, protoplasts were concentrated to $2 \times 10^6$ protoplasts per 200 µl of 0.6 MM buffer (0.6 M D-mannitol, 5 mM MES, pH 5.8). For inoculation, 200 µl of protoplasts in 50 ml disposable conical centrifuge tubes were mixed with either 30 µl of CTV *in vitro* transcribed clonal SP6 transcripts or 100 µl of sap from infected protoplasts, to which 500 µl of a solution of PEG 1540 (Polyscience, Inc.) [53.4 ml PEG 1540, 100 ml 0.75 MMC (0.75 M D-mannitol, 6.25 mM MES, 3.75 mM $CaCl_2$, pH 5.8), 164.6 ml 0.5 M $CaCl_2$] was added. The tube was immediately capped and rocked gently by hand for 20 s to mix the contents. The inoculation reaction was stopped by the addition of 5 ml of 0.6 MMC (0.6 M D-mannitol, 10 mM $CaCl_2$, 5 mM MES, pH 5.8). The protoplasts were allowed to incubate for 10 min at room temperature and were washed in Aoki and Takebe (1) culture medium (0.6 M D-mannitol, 5 mM MES, pH 5.8, with 1× Aoki and Takebe salts) containing a

1:150 dilution of Sigma (St. Louis, MO, USA) 100× Antibiotic/Antimycotic solution. Final resuspension of the protoplasts was in 3 ml and the volume was split between 2 wells of Falcon 6-well plates (Falcon #3036) that had been pre-coated with a layer of 0.6 M D-mannitol/1% agarose. Protoplasts were incubated at 26°C under constant illumination for four days. Protoplasts from one well were extracted immediately for RNA analysis, while the contents of the other well were pelleted, flash-frozen in liquid nitrogen, and stored at -80°C as a source of virus for future viral amplification via protoplast inoculations and passaging.

**Extraction of total RNA and virions from protoplasts.** For RNA analysis, $1.0 \times 10^6$ protoplasts were suspended in 500 µl Buffard buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 9.0, plus 2% SDS), extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with three volumes of 95% EtOH in the presence of 10% 3 M sodium acetate. For extraction of virions for inoculum to further amplify virions in protoplasts or for plant inoculations, frozen protoplast pellets in microfuge tubes were thawed in an ice bath and either 100 µl (for a single inoculation of protoplasts) or 200 µl (for two inoculations of protoplasts) of 40 mM $KH_2PO_4$, pH 7.4, with 5% sucrose was added for extraction of virus. The microcentrifuge tubes containing the resuspended protoplast pellets were gently flicked by hand every 10-15 min over a period of 1 to 1.5 h to release the virus into the buffer, and then centrifuged at 4,000 rpm for 5 min to clarify the extract.

**Ultracentrifugation of protoplast extracts through sucrose cushions.** Eleven milliliters of extract was pipeted into a SW41 Ultra-Clear™ ultracentrifuge tube (Beckman Instruments, Palo Alto, CA, USA), and a small portion was saved to test for virus by enzyme linked immunosorbent assay (ELISA). One milliliter of 70% sucrose was underlaid by inserting a long cannula through the extract with minimal mixing. The SW41 tubes were then centrifuged at 38,000 rpm for 75 min at 4°C. After centrifugation, the tubes were punctured at the base with a sharp tapered needle to collect fractions dropwise into premarked 1.5 ml microfuge tubes. The first fraction, Fraction 1 (F1), had a volume of 450 µl, and the three subsequent fractions (F2, F3, and F4) were collected at 100 µl each. A small sample of each fraction was diluted and tested by ELISA. Collected fractions were kept on ice or at 4°C until used.

**RNA Analysis.** Northern blots were used to determine whether inoculated constructs were increasing in titer with successive passages. After suspending the pelleted RNA in 50 µl of RNase-free water, Northern blot hybridization was performed as described (7). A digoxigenin-chemiluminescent detection system (Roche Diagnostics, Indianapolis, IN, USA) was used and all prehybridization, hybridization, washing and chemiluminsecent detection steps were performed according to the manufacturer's protocols. Riboprobes corresponding to the 900 nucleotides of the 3' end of the CTV genome were labeled with digoxigenin-labeled UTP and used to detect RNA bands. Initial film exposure was usually 1 hour. Shorter or longer exposures were used subsequently, depending on the intensity of the bands. Testing of serial dilutions was needed to make quantitative comparison of virus titer between preparations.

**ELISA.** Double Antibody Sandwich-Indirect (DAS-I) ELISA was used to estimate virus titer in protoplast extracts, to locate virus zones in sucrose cushions, and to confirm infection in inoculated plants. Procedures were as previously described (5). Anti-CTV rabbit polyclonal IgG coating antibody was used at 1 µg/

ml and the detecting antibody was anti-CTV Mab ECTV 172 (1:100K dilution). Extracts were tested at a 1/20 dilution unless otherwise noted. Assays were not considered positive unless $OD_{405}$ values were over twice the value of healthy extracts. Normal healthy extract values were typically 0.1 or less, and values for infected controls typically exceeded 2.0 at a 1/20 dilution.

**Stem Inoculation of Citrus Plants.** Two stem inoculation methods were used to inoculate citrus plants. For stem slash inoculations, 10-15 µl of inoculum was applied to a Feather No. 21 disposable scalpel blade and approximately 30 cross-cuts perpendicular to the axis of the stem were made while holding the blade at an angle to keep the drop on the blade. This was repeated so the stem was slashed on three sides. A total of 30-45 µl of inoculum was used per tree. For bark flap inoculation, four inverted "U" cuts approximately 2 to 3 cm long and 3 to 4 mm wide were made in the bark with a scalpel or budding knife. The bark at the top of the "U" was gently teased open about 3-4 mm, and 10-12 µl of inoculum was applied in the opening created. The bark flap was then pulled down the full length of the long cuts with forceps, so the inoculum flowed into the axis as the flap was formed. The flap was then put back into place and wrapped with grafting tape. Bark flaps were unwrapped after two weeks post-inoculation. Plants inoculated by either method were pruned 2 weeks post-inoculation to force a flush of new growth for ELISA testing.

## RESULTS AND DISCUSSION

Approximately 30 to 40 µl of inoculum is needed to inoculate each citrus seedling with the stem slash or bark flap procedures. Our goal was to inoculate five plants and obtain at least one infection. To produce approximately 200 µl of a 10-fold concentrated extract required production of approximately 2 ml of protoplast extract with a virion concentration similar to that found in infected bark tissue.

**Optimizing virus production in protoplasts.** The first step was to increase the very small virion population produced by the initial transcript inoculation via inoculation of a fresh batch of protoplasts. Because of the relatively high efficiency of protoplast inoculation using intact virions, an increased number of protoplasts were infected in this second inoculation, resulting in an increase in virus titer. Virus production was further increased in subsequent inoculations as illustrated in Fig. 1. Virus titer in protoplasts usually increased markedly between the 3rd to 5th passages. A critical step in this phase was using Northern blot analysis to monitor the increase of virus titer through successive passages, as shown in Fig. 2. The titer obtained in each passage is a function of the percentage of protoplasts that are infected, and the subsequent level of viral replication in those protoplasts; therefore, consistent and reproducible production of high quality protoplasts was especially critical in the initial passages. In the initial passages, the entire virus yield from the current infection cycle was needed to inoculate one new batch of protoplasts. In later passages, as virus yields increased, inoculation of extra batches of protoplasts was possible without a decrease in the virus titer per batch. This allowed a reservoir of frozen infected protoplasts to be maintained as an additional inoculum source for future passages and also to serve as a reserve in the event subsequent passages were not successful or were lost due to contamination. Generally, constructs were passaged through 4 to 10 successive cycles to achieve virus titers similar to that of protoplasts inoculated directly with infectious sap from CTV-infected trees. Passaging of a construct was considered to be successful when this level of amplifica-

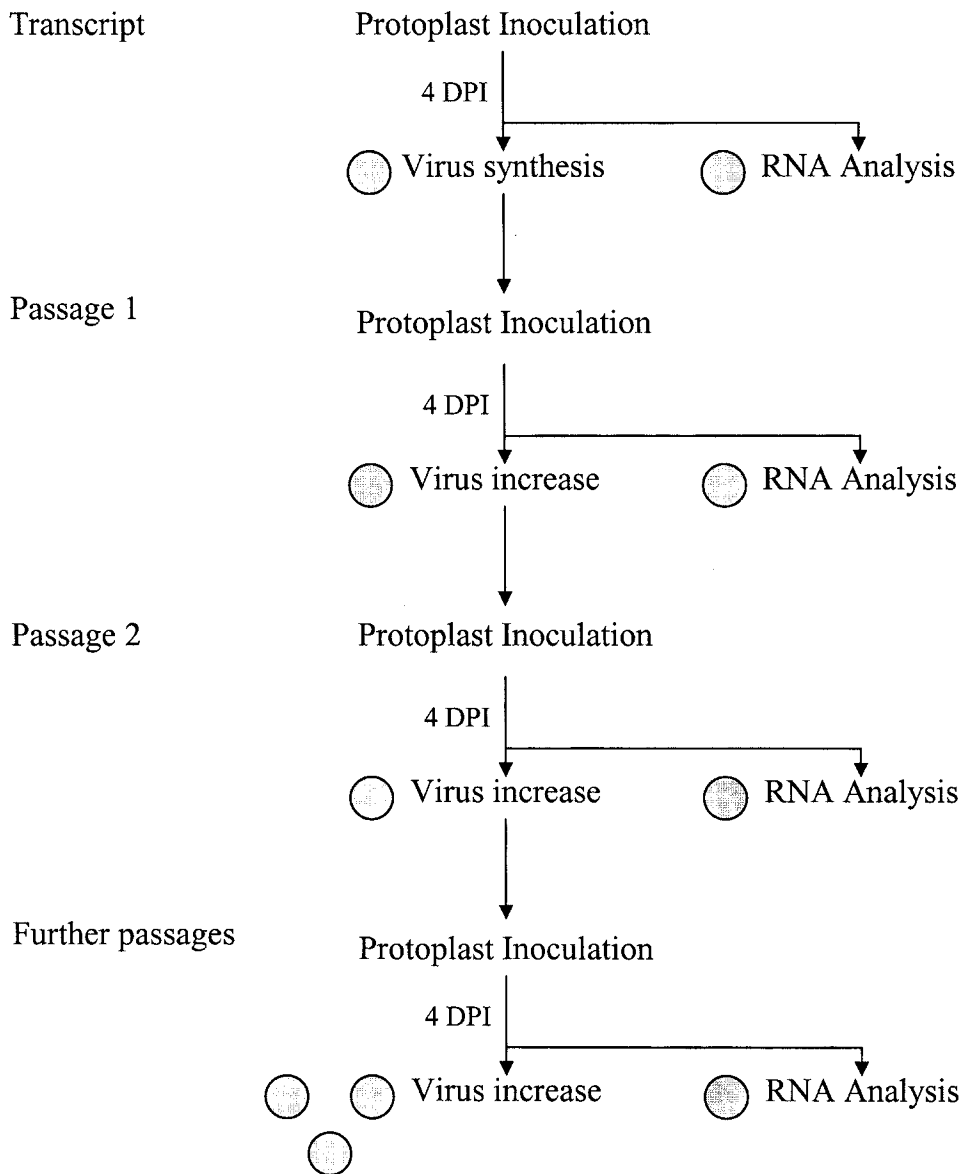


**Fig. 1. Flow chart illustrating the procedure of protoplast passaging. The circles represent harvested wells of *Nicotiana benthamiana* protoplasts, with each well containing 1 million protoplasts. Note at the bottom of the chart that as the amount of available virus increases, so does the total number of inoculated protoplasts. DPI is 'days post inoculation'.**

tion was achieved or when the virus titer could not be increased by further passages.

**Increasing inoculum supplies in protoplasts.** Once the titer was optimized, it was necessary to infect a large number of protoplasts to create a sufficient volume of extract for further concentration. The goal was to inoculate a final set of nine to twelve protoplast batches (18 to 24 million protoplasts) that would all yield maximally amplified amounts of virus. Extracts were tested by ELISA to verify that protoplasts yielded virus titer comparable

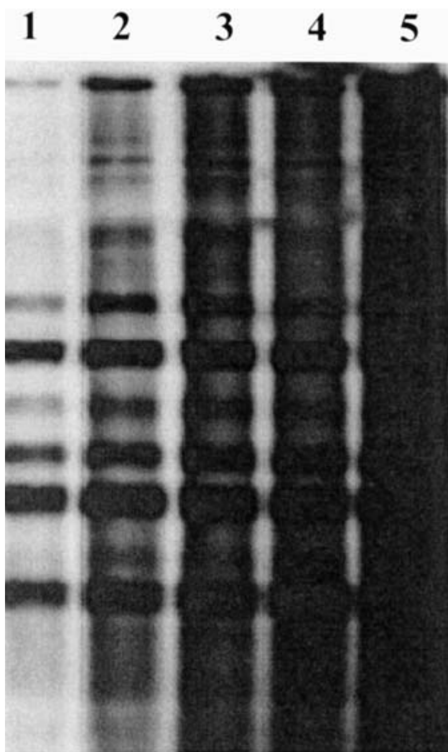


**Fig. 2. Northern blot showing the replication of *in vitro* transcripts in *Nicotiana benthamiana* protoplasts (Lane 1), followed by the amplification of progeny virus through four successive passages (Lanes 2 through 5), as described in Fig. 1.**

to those of protoplasts infected with sap from CTV-infected trees. At these higher concentrations of virus, 10-fold dilutions of extracts were also tested to more accurately estimate concentrations.

At this point, while we could infect citrus plants using highly amplified protoplast extracts with high titers, the percentage of plants that became infected was much lower than if the extract was concentrated and partially purified (Table 1).

**Concentration of virions by centrifuging through sucrose cushions.** Concentration and partial purification of CTV virions was accomplished by centrifugation of protoplast extracts through 70% sucrose cushions. This technique was used to avoid problems with resuspending the long filamentous particles from pellets without breakage. Under the conditions used, the virus particles accumulated in a zone about midway in the 70% cushion. The actual point of accumulation was determined by testing multiple fractions by ELISA and locating the leading (bottom) edge. Routinely, four fractions were collected (Fig. 3). Fractions 1 (F1) removed the bulk of the virus-free portion of the cushion below the leading edge of the virus zone. F2 captured the leading edge of the virus zone (as evidenced by ELISA); F2 and F3 contained the majority of the infectious virions. Higher fractions also tested positively by ELISA (Fig. 3), but were expected to contain more broken, non-infectious particles. Normally F2 and F3 were pooled to create the 200 µl of inoculum needed for a five plant assay. However, fractions were assayed separately, or pooled in other configurations. ELISA values of the sucrose cushion fractions were used to confirm that a threshold level needed for infection had been achieved.

**Plant inoculation techniques and assays.** Bark flap and stem slash inoculation techniques were both used to inoculate citrus plants with partially purified virus. In one comparative study, bark flap inoculation provided 100% infection while stem slash yielded 60% (Table 1, construct A). However, in looking at all plant inoculations over time, there was little apparent difference in infection efficiency between the two techniques. A limitation of the bark flap inoculation technique is that it could only be performed on plants when the cambium was active, so the bark was pulled easily away from the stem. Bark flaps also needed to be wrapped after inoculation. Stem slashing did not have these requirements. Two weeks after inoculation, plants were trimmed to force a new flush of growth. At least two flushes of growth were monitored for occurrence of symptoms (Fig. 4). The initial ELISA for detect-

TABLE 1
INFECTION RATES IN ALEMOW PLANTS INOCULATED WITH UNPURIFIED EXTRACTS OR CONCENTRATED SUCROSE CUSHION FRACTIONS FROM *NICOTIANA BENTHAMIANA* PROTOPLASTS INFECTED WITH THREE DIFFERENT CTV CLONAL CONSTRUCTS

| Construct | Inoculum | Inoculation Method | Infected/Total | % Infection |
|---|---|---|---|---|
| A | Unpurified | Bark Flap + Stem Slash | 1/5 | 20 |
| A | Partially purified | Bark Flap | 5/5 | 100 |
| A | Partially purified | Stem Slash | 3/5 | 60 |
| B | Unpurified | Bark Flap + Stem Slash | 1/5 | 20 |
| B | Partially purified | Bark Flap | 5/5 | 100 |
| C | Unpurified | Bark Flap + Stem Slash | 1/5 | 20 |
| C | Partially purified | Bark Flap | 5/5 | 100 |

ing infection generally used maturing young leaf petiole or bark tissue at the end of the second flush of new growth. If no symptoms appeared and ELISA tests remained negative for CTV six months after inoculation, plants were considered uninfected and were discarded.

The methodology described has been routinely used for a large number of new clones. These include various deletions, insertions and hybrid constructs between several CTV genotypes. Overall plant infection rates have increased from 8% to 85% (160 infected plants in 189 total attempts) and 38 new CTV constructs have been introduced into citrus plants in the past 21 mo. Previous work had only established seven constructs into citrus in three years. As infection of citrus plants has become more predictable and reliable, only five or fewer inoculated plants are needed to provide a high probability that an infection with a construct will be established. This has reduced requirements for greenhouse space and time, and also reduced ELISAs needed to evaluate inoculated plants. In several cases, high rates of infection have been achieved even though the ELISA values for the sucrose cushion fractions were somewhat less than the threshold goal established. It is probable that the gentle extraction method used to release virions from protoplasts results in a high percentage of intact infectious virions.

Being able to estimate and quantify the changes in virus titer during the protoplast passaging was important. Once a substantial amplification had occurred, comparing virus titer by either ELISA or Northern blot analysis required testing of serial dilutions to measure relative differences between CTV clonal construct-infected and CTV bark sap-infected protoplasts. This work confirmed the original hypothesis that lack of sufficient titer was responsible for low infection rates in plants, and that a threshold level of sufficient inoculum was required for high percentages of plant infection.

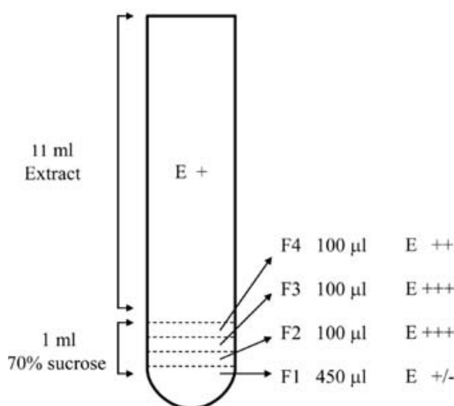


**Fig. 3. Location and volumes of sucrose cushion fractions (F1-F4) and extract volume from centrifugation of virus through a sucrose cushion. Location of virus indicated by a +/- system for relative ELISA (E) values.**

**Fig. 4. Veinal chlorosis and leaf cupping symptoms in Alemow infected with a CTV cloned construct.**

In spite of the significant improvement in infection rates, establishing cloned CTV constructs in citrus remains a technically challenging and time-consuming process. Infection of protoplasts by transcript inoculation still remains at the minimum threshold level needed to get detectable production of virions. The multiple amplification cycles with RNA analysis each require about one week of work. Various factors affect infection, virus yield, and survival of protoplasts. These factors include the quality of *N. benthamiana* plants used for production of protoplasts, microbial contamination of protoplasts, and growth chamber failure. Unexplained decreases in titer also can occur midway in a sequence of successive passages. Preservation of an inoculum backup as soon as possible is extremely important as it can save weeks of work should a passage fail.

In addition, the amplification procedure described only works with constructs that are capable of replication in *N. benthamiana* protoplasts and are capable of assembly. The CTV T30 genotype does not replicate in *N. benthamiana* protoplasts, so studies with T30 will rely on the development of a competent citrus protoplast system for subsequent plant infection (M. R. Albiach-Martí, unpublished data).

Research is still continuing to find a more direct approach to establish infectious CTV clones in citrus, such as *Agrobacterium*-mediated infection (6) and DNA bombardment.

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