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## *Citrus Tristeza Virus*: Survival at the Edge of the Movement Continuum<sup>∇</sup>

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**Systemic invasion of plants by viruses is thought to involve two processes: cell-to-cell movement between adjacent cells and long-distance movement that allows the virus to rapidly move through sieve elements and unload at the growing parts of the plant. There is a continuum of proportions of these processes that determines the degrees of systemic infection of different plants by different viruses. We examined the systemic distribution of *Citrus tristeza virus* (CTV) in citrus species with a range of susceptibilities. By using a “pure” culture of CTV from a cDNA clone and green fluorescent protein-labeled virus we show that both cell-to-cell and long-distance movement are unusually limited, and the degree of limitation varies depending on the citrus host. In the more-susceptible hosts CTV infected only a small portion of phloem-associated cells, and moreover, the number of infection sites in less-susceptible citrus species was substantially decreased further, indicating that long-distance movement was reduced in those hosts. Analysis of infection foci in the two most differential citrus species, *Citrus macrophylla* and sour orange, revealed that in the more-susceptible host the infection foci were composed of a cluster of multiple cells, while in the less-susceptible host infection foci were usually single cells, suggesting that essentially no cell-to-cell movement occurred in the latter host. Thus, CTV in sour orange represents a pattern of systemic infection in which the virus appears to function with only the long-distance movement mechanism, yet is able to survive in nature.**

Viruses that infect higher plants share a number of common principles with animal viruses. Among those are virion morphology and strategies for replication and expression of their genomes. However, to establish a productive infection in a host the plant virus needs to be able to move throughout a plant from an initially infected cell. Success depends upon compatible interactions between viral and host factors. Generally, systemic movement is thought to involve two distinct processes: cell-to-cell movement, which according to our definition is a process that allows the virus to transverse the cell wall between adjacent cells, and long-distance movement, which is a process that allows the virus to enter the sieve element from an adjacent nucleated cell and rapidly move through the connected sieve elements, followed by its exit into another adjacent phloem-associated cell at a distal region of the plant. A major obstacle for the spreading virus is to cross the boundaries represented by the cell wall. For this purpose most viruses utilize specific virus-encoded movement proteins as well as some host proteins that facilitate their translocation through plasmodesmata channels. The viral proteins and their interactions with the host during cell-to-cell movement are fairly well known (reviewed in references 26, 43, and 45). However, the mechanisms of long-distance transport and factors that aid

virus entrance into phloem tissue, further vascular movement, and unloading from phloem are much less understood.

Different viruses utilize different ratios of cell-to-cell and long-distance movement, which results in significant differences in the extent and patterns of systemic invasion of their hosts. Often the virus-host interaction results in no disease when the virus is able to replicate in initially infected cells but is not able to move throughout the plant, which is considered a nonhost of the virus. One ultimate example of spread is exemplified by the well-studied system of *Tobacco mosaic virus* (TMV) in tobacco, where the virus moves efficiently, infecting most of the cells throughout the entire plant. This sequence typically results from cell-to-cell movement from an initially infected cell through plasmodesmata to neighboring cells until the virus reaches phloem cells. Then, long-distance movement allows the virus to rapidly move through sieve elements and unload at the growing parts of the plant, where further cell-to-cell movement from phloem-associated cells allows the virus to invade most of the cells of these distal plant organs. Once the virus approaches the growing point via long distance movement, continued cell-to-cell movement parallels plant growth to maintain the systemic infection. Other patterns of movement allow a range of more limited systemic infection of plants. Some viruses spread systemically throughout plants but are confined mainly to cells associated with phloem. Therefore, in nature the virus is usually introduced directly into phloem-associated tissues by a vector, often an insect. The virus then is able to move normally by long-distance movement but is limited in cell-to-cell movement to nearby phloem-associated cells.

Not only must the virus have the capacity to replicate and move in a particular plant host, but also it must have the ability to escape from the host's surveillance system. Along with

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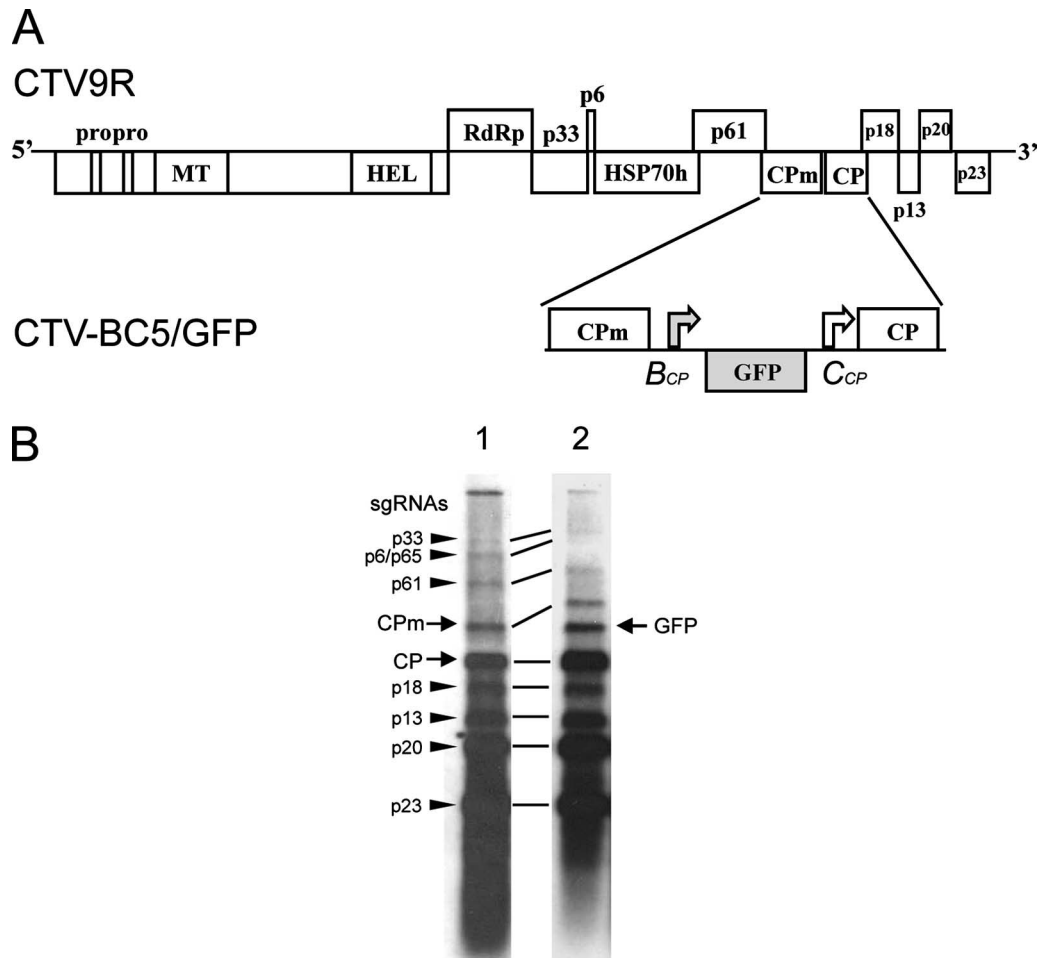


FIG. 1. (A) Schematic diagram of the genome organization of wild-type CTV (CTV9R) and its derivative encoding GFP. The open boxes represent ORFs and their translation products. PRO, papain-like protease domain; MT, methyltransferase; HEL, helicase; RdRp, an RNA-dependent RNA polymerase; HSP70h, HSP70 homolog. Bent arrows indicate positions of BYV ( $B_{CP}$ ) or CTV CP ( $C_{CP}$ ) sgRNA controller elements. Inserted elements are shown in gray. (B) Replication of CTV9R (lane 1) or CTV-BC5/GFP (lane 2) in *N. benthamiana* mesophyll protoplasts inoculated with virions extracted from infected *C. macrophylla* seedlings. Total RNA was isolated from protoplasts 4 days postinoculation. Northern blot hybridizations were carried out using CTV 3' positive RNA strand-specific riboprobe. Positions of sgRNAs are shown.

movement functions, viruses also encode another group of factors termed silencing suppressors that counteract the RNA interference plant defense system to allow a systemic infection to be established and maintained (33, 35, 44). Mutations of viral suppressor genes often result in reduction or prevention of systemic infection (9, 21, 32). In fact, the tissue limitations can be due to the plant defense system. Experiments with coinoculation of phloem-limited viruses, the polerovirus *Potato leafroll virus*, or bipartite geminiviruses *Bean golden mosaic virus* and *Abutilon mosaic virus* with certain other viruses resulted in alleviation of their phloem limitation, which demonstrated that perhaps confinement to phloem could be explained by lack of necessary factors that enable these viruses to unload into mesophyll tissue and move further cell to cell and/or by lack of the mechanisms to overcome the plant defense system outside the phloem (6, 7, 27, 36, 46).

*Citrus tristeza virus* (CTV) is limited to phloem-associated cells in citrus trees. It is the largest and most complex member of the *Closteroviridae* family, which contains viruses with mono-, bi-, and tripartite genomes (1, 5, 11, 12, 20). Members

of this family are transmitted by different types of insects: aphids, whiteflies, and mealybugs. CTV has long flexuous virions (2,000 nm by 10 to 12 nm) encapsidated by two coat proteins and a single-stranded RNA genome of approximately 19.3 kb. The major coat protein (CP) encapsidates about 97% of the genomic RNA, while the minor coat protein (CPm) covers the rest of the genome at its 5' end (13, 41). The RNA genome of CTV encodes 12 open reading frames (ORFs) (18, 28) (Fig. 1). ORFs 1a and 1b are expressed from the genomic RNA and encode polyproteins required for virus replication. Ten 3'-end ORFs are expressed by 3'-coterminal subgenomic RNAs (sgRNAs) (17, 19) and encode the following proteins: CP, CPm, p65 (HSP70 homolog), and p61, which are involved in assembly of virions (38); a hydrophobic p6 protein with a proposed role in virus movement (12, 42); p20 and p23, which along with CP are suppressors of RNA silencing (25); p33, p13, and p18, whose functions remain unknown.

The host range of CTV generally is limited to citrus species and relatives, and the different species exhibit differential degrees of susceptibility to CTV infection (8; S. M. Garnsey,

personal communication). On the other hand, a number of citrus relatives, including *Poncirus trifoliata*, *Swinglea glutinosa*, and *Severinia buxifolia*, demonstrate immunity to infection by most CTV isolates (15, 47, 48, 49). Since CTV can replicate in protoplasts isolated from these plants, the resistance at a whole-plant level possibly results from a lack of virus movement (2). Experiments with the resistant genotype used as an interstock grafted between two susceptible genotypes showed that the virus moves though the resistant interstock unimpeded but does not multiply detectably in the resistant plant (S. M. Garnsey, personal communication). Therefore, it appears likely that the virus is unable to egress from the sieve elements into adjacent phloem cells of the resistant genotype. It is possible that the gradient of susceptibility of different citrus species to CTV is related to the ability of the virus to move into and infect cells surrounding sieve elements.

The objective of this work was to examine the systemic distribution of CTV in citrus species with a range of susceptibility and to attempt to interpolate movement mechanisms responsible for the distribution. We show that CTV in citrus trees generally follows similar patterns, but the degrees of both cell-to-cell and long-distance movement are more limited than most systems that have been described, and the limitation varies depending on the citrus host. In all hosts, long-distance movement appears to be limited to relatively few initial infection sites. In the more-susceptible citrus species, CTV also has limited cell-to-cell movement that produces small clusters of infected cells. However, in less-susceptible citrus species, it appears that no cell-to-cell movement occurs. The virus is able to exit sieve elements but cannot spread to adjacent cells, resulting in infection of isolated single cells. Thus, here we demonstrate a new pattern of systemic infection in which the virus appears to function with only the long-distance movement mechanism, yet is able to survive in nature. Elucidation of this process aids in understanding how the virus survives long term (up to about 100 years) in infected trees and how it spreads to other trees.

#### MATERIALS AND METHODS

**Virus constructs, amplification of virions in *Nicotiana benthamiana* protoplasts, and inoculation of citrus trees.** Full-length cDNA constructs were used in this study: pCTV9R, a cDNA clone of T36 strain of CTV (37, 40), and constructs containing insertion of green fluorescent protein (GFP) ORF in the region between the CPm and CP genes in the CTV genome, pCTV-BC5/GFP (14) and CTV9-GFPC3 (42). SP6 RNA polymerase-derived transcripts of CTV cDNAs linearized with NotI restriction endonuclease were used for transfection of *N. benthamiana* mesophyll protoplasts according to the procedure described by Satyanarayana et al. (37). Protoplasts were harvested at 4 days postinoculation and stored at  $-70^{\circ}\text{C}$  for subsequent protoplast passage of virions. Passaging of virions up to 11 successive cycles in protoplasts for amplification of the virus was done as described previously by Satyanarayana et al. (38). Accumulation of the virus was monitored by Northern blot hybridization of the total RNA isolated from protoplasts with a 3' positive-stranded CTV RNA-specific riboprobe (37).

Amplified progeny virions from the final passages in protoplasts were extracted and concentrated by sucrose cushion centrifugation, and the concentrated virions were used for mechanical "bark flap" inoculation of small trees of *Citrus macrophylla* Wester as described by Robertson et al. (34). Infected trees were later used as a source of virus inocula for subsequent graft inoculations of young *C. macrophylla* trees as well as other citrus species: Mexican lime [*Citrus aurantifolia* (Christm. Swing)], Madam vinous sweet orange [*Citrus sinensis* (L.) Osbeck], sour orange (*Citrus aurantium* L.), and Duncan grapefruit (*Citrus paradisi* Macf.).

**Serological assays.** A double antibody sandwich indirect enzyme-linked immunosorbent assay (ELISA) was performed as described previously using anti-

bodies specific to CTV virions (16) to confirm infection in inoculated plants and compare titers of virus accumulation in different citrus species. Purified immunoglobulin G from rabbit polyclonal antiserum CTV-908 (1  $\mu\text{g}/\text{ml}$ ) was used as a coating antibody. ECTV172, a broadly reactive CTV monoclonal antibody, was used as a detecting antibody.

**Examination of fluorescence in citrus plants infected with GFP-tagged CTV.** Samples of bark tissue from CTV-BC5/GFP-infected trees were examined for GFP fluorescence at different time points beginning at 6 weeks after inoculation using a Zeiss Stemi SV 11 UV fluorescence dissecting microscope (Carl Zeiss Jena, GmbH, Jena, Germany) with an attached Olympus Q-color 5 camera (Olympus America, Inc., Center Valley, PA). More detailed observation of the infection foci in infected *C. macrophylla* and sour orange trees was performed using a confocal scanning microscope (Leica TCS SL; Leica Microsystems, Inc., Exton, PA).

**Light and transmission electron microscopy and immunogold labeling.** Petioles of young leaves of *C. macrophylla* and Mexican lime trees and bark tissue samples of *C. macrophylla* and sour orange trees were harvested for electron microscopy and light microscopy studies, respectively, 6 to 8 weeks after inoculation with CTV9R. Tissue samples were fixed in phosphate buffer containing 1.8% paraformaldehyde and 0.25% glutaraldehyde and embedded in LR White resin (London Resin Company, Hampshire, United Kingdom). Procedures for the specimen fixation and embedding, as well as immunogold labeling, were carried out as described by Zhou et al. (50). The immunogold labeling of sections (semithin 2- $\mu\text{m}$  sections for light microscopy or ultrathin 80- to 90-nm sections for electron microscopy) of LR White-embedded samples was performed using purified immunoglobulin G from rabbit polyclonal antiserum CTV-908 (1  $\mu\text{g}/\text{ml}$ , 1:1,000 dilution) as primary antibody and goat anti-rabbit-10-nm gold conjugate (1:25 dilution; Sigma). For light microscopy studies gold labeling was enhanced by incubating with silver enhancing solution (BioCell Research Laboratories, Cardiff, United Kingdom) as described by Ding et al. (10). Specimens were observed using a Morgagni 268 transmission electron microscope (FEI, The Netherlands) or Leitz LaborLux S light microscope (Leica, Germany).

#### RESULTS

**Different citrus hosts support different titers of CTV.** The dogma is that CTV titers vary considerably in different citrus species (8; S. M. Garnsey, personal communication). (It should be noted that citrus taxonomy is complex. For example, sweet orange [*C. sinensis*] and grapefruit [*C. paradisi*] are classified as species but now are known to be hybrids instead.) Titers also vary with different isolates of virus, which generally are populations composed of a mixture of viral genotypes whose sequences fit into different sequence groups that we now consider strains. Some populations accumulate to higher levels than others in the same type of host. Also, measurements of viral titers have been done in different countries, with different environments, and in different citrus varieties. So, although it has been an accepted phenomenon that some citrus varieties tend to support higher titers of CTV while other varieties support lower titers, the variation among different isolates and different measurements has greatly confounded this understanding.

The development of an infectious cDNA clone of the T36 strain of CTV (37) allowed us to obtain a "pure" culture of the virus in citrus plants (39) using an inoculum that originated from RNA transcripts of the cDNA clone. The recombinant CTV (CTV9R) culture has remained a uniform population during the first 6 years of infection of citrus trees, which was confirmed by resequencing of the entire genome (Z. Xiong et al., unpublished data). We examined the susceptibility of different citrus species to this strain of CTV in five *Citrus* spp.: *C. macrophylla*, Mexican lime, Madam vinous sweet orange, sour orange, and Duncan grapefruit. Three plants of each were graft inoculated with CTV9R. CTV is usually assayed in the first flush of new leaves that develops after graft inoculation of



TABLE 1. Analysis of CTV accumulation in five citrus species

Exptl group	CTV titer <sup>a</sup> in:				
	<i>Citrus macrophylla</i>	Mexican lime	Sweet orange Madam Vinous	Duncan grapefruit	Sour orange
CTV9R infected	3.08 ± 0.015	2.95 ± 0.021	0.92 ± 0.065	0.54 ± 0.030	0.32 ± 0.015
Healthy	0.10 ± 0.004	0.09 ± 0.003	0.08 ± 0.008	0.09 ± 0.009	0.07 ± 0.008

<sup>a</sup> CTV9R-infected trees were assayed at 6 weeks postinoculation by double antibody sandwich indirect ELISA using CTV-specific 908 IgG as trapping antibody at a 1- $\mu$ g/ml concentration and ECTV 172 monoclonal antibody as detecting antibody at a 1:50,000 dilution. ELISA values ( $A_{405}$ ) are averages for three plants  $\pm$  standard deviations.

the lower trunk. At 6 weeks after inoculation, the upper leaves were harvested, extracted, and assayed by ELISA with CTV-specific antibodies. Higher titers of the virus were detected in *C. macrophylla* and Mexican lime trees, while in sour orange and grapefruit titers of the virus were significantly lower. Sweet orange showed intermediate levels of CTV accumulation (Table 1). These data indicated that these species represent a good selection of citrus hosts with different degrees of virus infection.

#### CTV infects only a proportion of phloem-associated cells.

Previous studies on cytopathology and ultrastructure of CTV in citrus plants showed that the virus accumulates preferentially in the phloem parenchyma and to a lesser degree in companion cells (4, 22, 23, 50). CTV virions were also found occasionally in sieve elements. Although symptoms and cellular reactions caused by the virus are different depending on citrus species and isolates of virus, so far there is little information about virus movement and distribution in those hosts. A recent cytopathological study conducted using transmission electron microscopy also did not reveal any differences in virus distribution between different hosts (50). We chose to further examine the quality and quantity of cells in different hosts to attempt to better understand possible reasons for different titers.

We first examined the distribution of CTV in the more susceptible hosts, *C. macrophylla* and Mexican lime, by transmission electron microscopy of thin sections of leaf petioles of trees infected with CTV9R. Usually CTV induces several types of inclusions in infected phloem parenchyma and companion cells. Those are viral arrays, fibrous inclusions, and cytoplasmic vesicles and have been described in detail previously (50). These characteristic ultrastructural changes make it relatively easy to differentiate the infected cells from uninfected ones using the electron microscope. To further improve the detection of infected cells, we also used immunogold labeling with T36 CTV-specific antibodies, which react with virions, making them readily distinguishable from P-proteins and other thread-like structures in infected phloem cells. Figures 2 and 3 show transmission electron micrographs of phloem cells in petioles of infected *C. macrophylla* and Mexican lime plants, respectively. Figures 2A and 3A show groups of cells at lower magnification, while Fig. 2B, C, and D and Fig. 3B and C show areas of infected cells at higher magnification to visualize immunogold labeling. Both figures demonstrate that even in the cases of these two species, which usually have the highest virus concentrations, the virus infects only a small proportion of phloem-associated cells. In general, we found approximately 2 to 3 infected cells per 10 to 20 cells in a viewed area. Consid-

ering that some species of citrus support much lower titers of the virus, including sour orange, which has less than 1/10 the virus concentration of *C. macrophylla* or Mexican lime (Table 1), we would expect to find an even lower number of infected cells. This low proportion of infected cells, in addition to the difficulty of obtaining statistically relevant sample sizes using electron microscopy, diminished the usefulness of this approach to reveal the differences of CTV interactions with the various citrus hosts.

**Distribution of CTV varies in different citrus hosts.** Because of the limitations of the above approach, as an alternative method to examine the distribution of CTV in different citrus species, we used GFP-tagged CTV (14), which allowed observations to be made using a fluorescence dissecting microscope of many more samples from different plants and allowed scanning of larger areas of infected tissues. The CTV-based vector, CTV-BC5/GFP (Fig. 1), contained the GFP ORF inserted into the virus genome as an extra gene (14). The initial work of characterizing the vector was in *C. macrophylla*. At 5 weeks after inoculation, the GFP-tagged virus was found to have replicated and moved systemically into young leaves of the tree. The vector has been unusually stable, continuing production of GFP in citrus trees for 5 years so far. The biological characteristics of the CTV-GFP vector in citrus trees were essentially identical to those of wild-type CTV, with both viruses exhibiting similar time intervals for establishing systemic infections, similar symptoms produced in infected plants, and appearing to be equally competitive when inoculated simultaneously into the same tree (14). These features make the GFP-expressing CTV-based construct highly suitable for studying virus distribution in citrus trees.

Small trees of *C. macrophylla*, Mexican lime, sweet orange, sour orange, and grapefruit were graft inoculated with CTV-BC5/GFP. Six weeks after inoculation, the new flush of leaves and stems of the infected trees was assayed for GFP fluorescence.

In rapidly growing citrus trees, the bark slips, which means that the bark freely pulls away from the wood, separating at the cambial layer, leaving the xylem cells with the woody trunk and the phloem-associated cells on the inside of the excised bark. This allows observation of the phloem-associated cells on the inside of the bark piece by using a dissecting fluorescence microscope. Examination of *C. macrophylla* bark tissue from several young flushes revealed an intermittent pattern of distribution of GFP fluorescence in the phloem-associated cells (Fig. 4). Stretches of cells exhibited fluorescence throughout the bark, but most cells on the phloem surface failed to exhibit fluorescence, demonstrating that only a portion of the phloem-

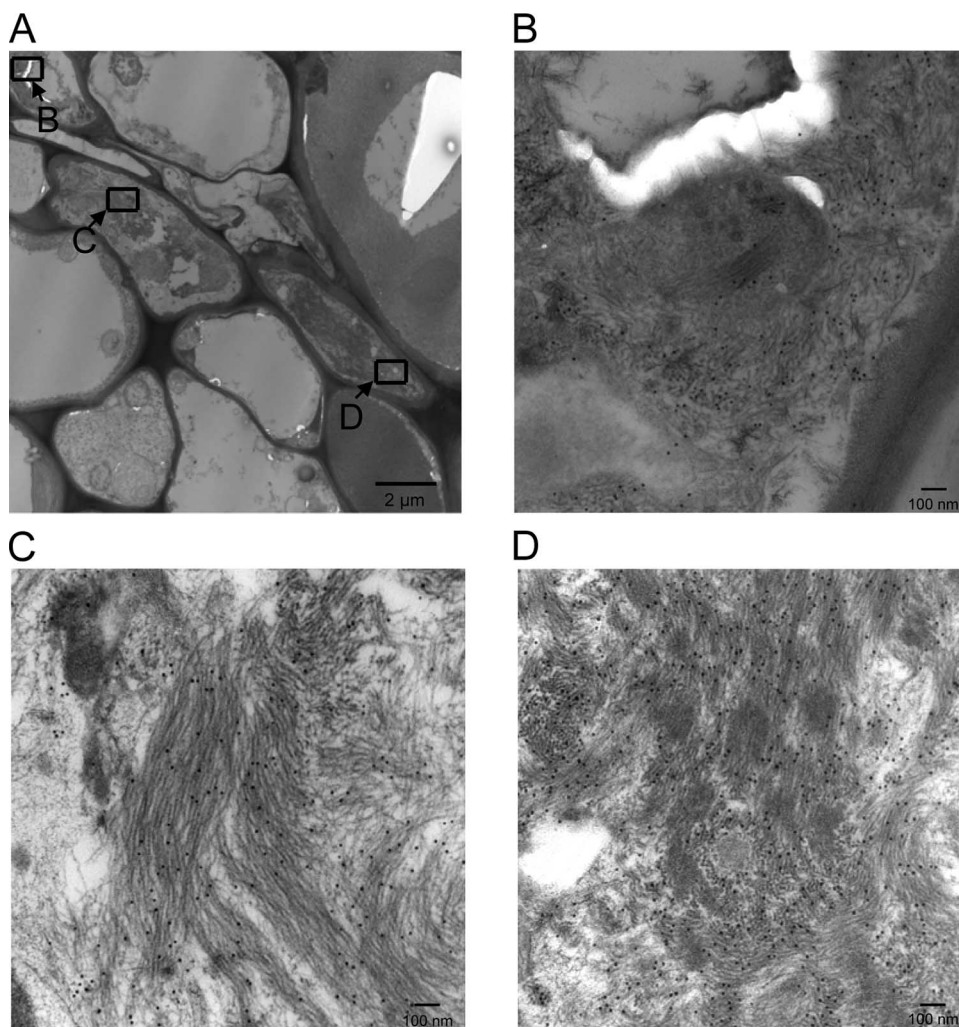


FIG. 2. Transmission electron micrographs showing phloem cells in a petiole of *C. macrophylla* infected with CTV9R. (A) Groups of phloem cells at lower magnification. Infected cells are indicated with letters B, C, and D, corresponding to images in subsequent panels. (B to D) Areas from the cells shown in panel A at higher magnification. Viral arrays were labeled with polyclonal CTV-specific antibodies used as primary antibodies and secondary antibodies conjugated with 10-nm gold particles. No labeling was detected in noninfected cells.

associated cells became infected. Examination of bark from older flushes and leaf midribs and veins showed similar patterns of fluorescence. It is difficult to calculate the ratio of the infected phloem cells to the uninfected ones, but based on the observations of multiple bark pieces from infected *C. macrophylla* trees we estimate that less than 10 to 20% of cells fluoresced.

The distribution of GFP fluorescence in Mexican lime trees infected with CTV-BC5/GFP was similar to that in *C. macrophylla* trees (Fig. 4), which coincides with the similar ELISA values of CTV in these two hosts (Table 1). However, the patterns of fluorescence in sour orange and grapefruit trees were very different. Many fewer and smaller fluorescent spots were seen in the bark pieces from these trees. The reduced GFP fluorescence paralleled the reduced viral titers in these hosts. However, the intensity of fluorescence within individual infected cells in sour orange and grapefruit appeared to be similar to that in infected cells of *C. macrophylla* and Mexican lime trees, suggesting that the reduced titers of virus revealed

by ELISA were correlated with fewer cells infected. Observations of bark tissue from several infected sweet orange trees showed that the frequency of fluorescent foci in these plants was less than that in *C. macrophylla* and Mexican lime but much higher than that in sour orange or grapefruit. In all five species, the pattern of distribution of CTV-induced fluorescence was correlated with the ELISA values of virus titer in those hosts (Fig. 4; Table 1).

**Analysis of infection foci in duplex plants and grafted bark patches.** The numbers of fluorescent sites were much lower in grapefruit and especially in sour orange than in *C. macrophylla* and Mexican lime. This may have resulted from a decreased ability of the virus to move from sieve elements into adjacent cells or a lower susceptibility of the adjacent cells. However, it also could result from lower inocula being generated within infected cells of these trees and less virus in the sieve elements to move into adjacent cells. We examined this possibility by two different approaches. The first was to examine “duplex” plants, in which side grafts from healthy sour orange plants

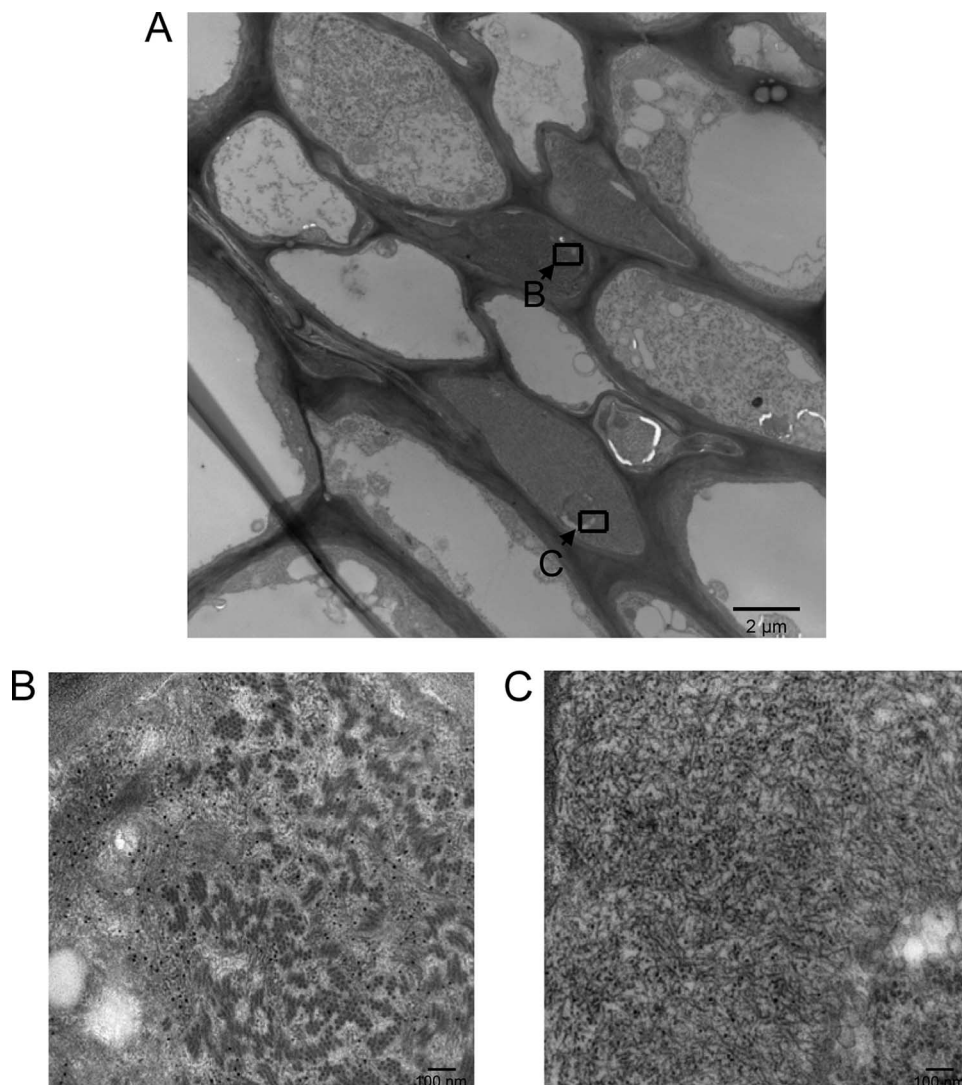


FIG. 3. Transmission electron micrographs showing phloem cells in a petiole of Mexican lime infected with CTV9R. (A) Groups of phloem cells at lower magnification. Infected cells are indicated with letters B and C, corresponding to images in those panels. (B and C) Areas from the infected cells shown in panel A at higher magnification. Masses of virions were labeled with polyclonal CTV-specific antibodies used as primary antibodies and secondary antibodies conjugated with 10-nm gold particles.

were put onto *C. macrophylla* seedlings infected with CTV-BCN5/GFP and new shoots of the sour orange and *C. macrophylla* were trained to grow in parallel from the same infected *C. macrophylla* rootstock. Thus, the inoculum source for both new shoots was identical. The second approach was to excise small squares of bark from the stems of *C. macrophylla* trees, replace them with bark pieces of an equal size from a sour orange tree, and allow the substituted bark patches to become grafted in place. Reciprocal grafts were made by replacing bark patches from *C. macrophylla* into sour orange trees. Two and 6 months after inoculation with GFP-expressing CTV, tissue from duplex plants and plants with bark patches was analyzed for GFP expression. In both cases very few fluorescent infection foci were found in sour orange tissue (Fig. 5). The *C. macrophylla* portion of the same trees appeared to be heavily infected and could have served as a continuous virus supply. Nevertheless, this did not affect the level of infection in sour

orange tissues, and the distribution of infection foci remained similar to that observed earlier in individual sour orange plants inoculated with CTV-BC5/GFP, demonstrating that the pattern of virus distribution in these hosts was not related to the amount of inoculum.

**Confocal microscopy of infection foci produced in *C. macrophylla* and sour orange.** We next examined the fluorescent foci in the two most differential citrus species, *C. macrophylla* and sour orange, by confocal microscopy to estimate the relative amounts of fluorescence per cell as an estimate of the level of virus replication per cell and determine how many cells occur in individual foci. In *C. macrophylla*, each infection focus was composed of multiple cells, ranging from 3 to more than 12 (Fig. 6). Strikingly, observations of sour orange tissues revealed an absence of cell clusters in sites of infection. Infection foci consisted of single cells (Fig. 6). These observations were made for many individual tissue samples from several indepen-



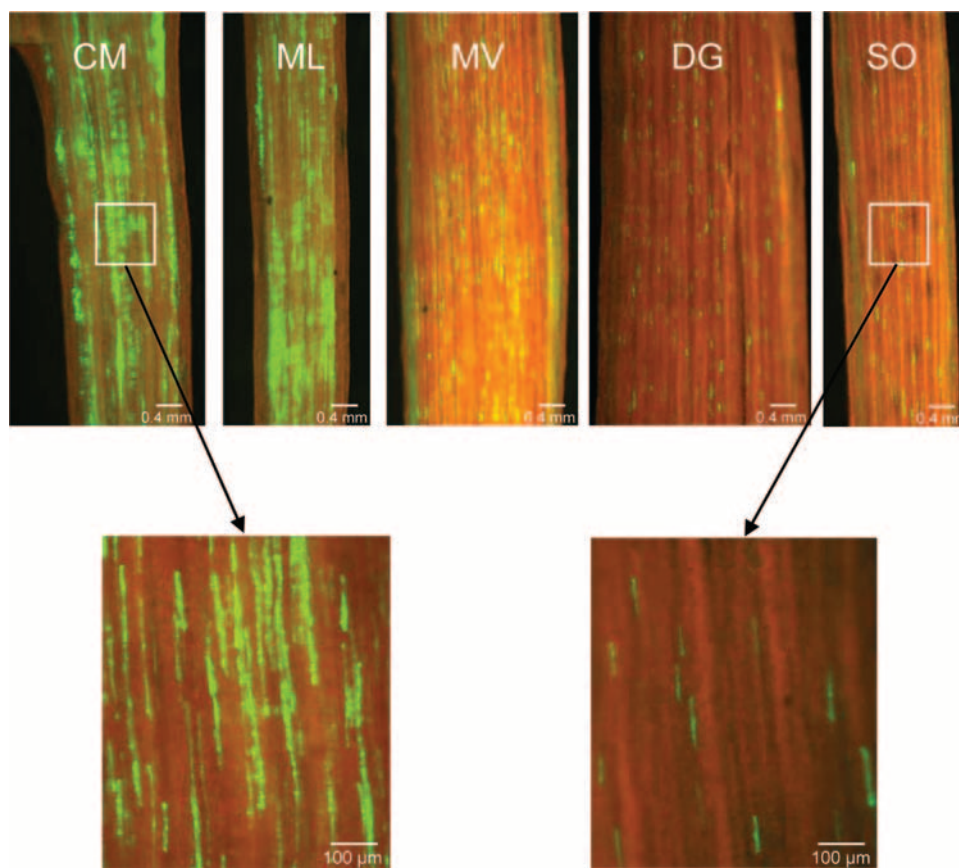


FIG. 4. Detection of GFP fluorescence in phloem-associated cells on the internal surface of bark of *C. macrophylla* (CM), Mexican lime (ML), sweet orange Madam Vinous (MV), Duncan grapefruit (DG), and sour orange (SO) trees infected with CTV-BC5/GFP at 6 weeks after inoculation (micrographs in the top row). Indicated areas of fluorescing cells in bark of *C. macrophylla* and sour orange are shown at higher magnification (bottom row) to demonstrate dramatic differences in virus distribution in these two species.

dent infected trees, and all of them showed similar results. However, the intensity of fluorescence in infected cells appeared to be similar in *C. macrophylla* and sour orange, suggesting that the reduced titer of virus in sour orange resulted mainly from a reduction in number of cells infected.

To corroborate the confocal microscopy of the GFP-labeled CTV, we utilized a light microscopy technique to examine tissues of these two citrus hosts infected with wild-type CTV. Tissue sections of fixed samples from infected trees were labeled with CTV-specific antibodies as primary antibodies and antibodies with attached gold particles as secondary antibodies. To visualize infected cells at the light microscopy level, labeling was further enhanced according to the silver enhancement technique. Figure 7 shows that groups of infected cells were found in *C. macrophylla* samples, while only single cells were present in sour orange tissues. Thus, patterns of cells infected with wild-type virus observed with the immunogold-silver enhancement technique in these two citrus species confirmed our findings with confocal microscopy of the GFP-labeled virus.

## DISCUSSION

Although the dogma has been that CTV accumulates to different amounts in different hosts, this has been confounded

by the use of different populations of virus, different hosts, and different environments. Use of a pure culture of CTV from a cDNA clone and the ability to label CTV with GFP allowed visualization of virus replication, accumulation, and distribution in different citrus species, clearly showing that some species were much more susceptible to the virus than others. In the more-susceptible species, *C. macrophylla* and Mexican lime, many more cells became infected. Infection sites consisted of clusters of 3 to 12 cells. In the less-susceptible species, sour orange and grapefruit, there were fewer infection sites and they usually were single cells. Sweet orange tended to be intermediate between these two extremes. Our interpretation is that systemic invasion of CTV begins when the virus enters sieve elements of the phloem, which transport the virus from some distal position in the direction of sugar movement (source to sink), after which at some point the virus exits into an adjacent cell, usually in stems and leaf veins of a new flush. We assume that the adjacent cell is a companion or phloem parenchyma cell, but this differentiation in citrus phloem is not readily apparent, especially when using confocal microscopy of GFP-labeled virus. We refer to this process as long-distance movement. We consider the movement of virus to fill the cluster of multiple cells as cell-to-cell movement.

The fewer infection sites in sour orange than in *C. macro-*



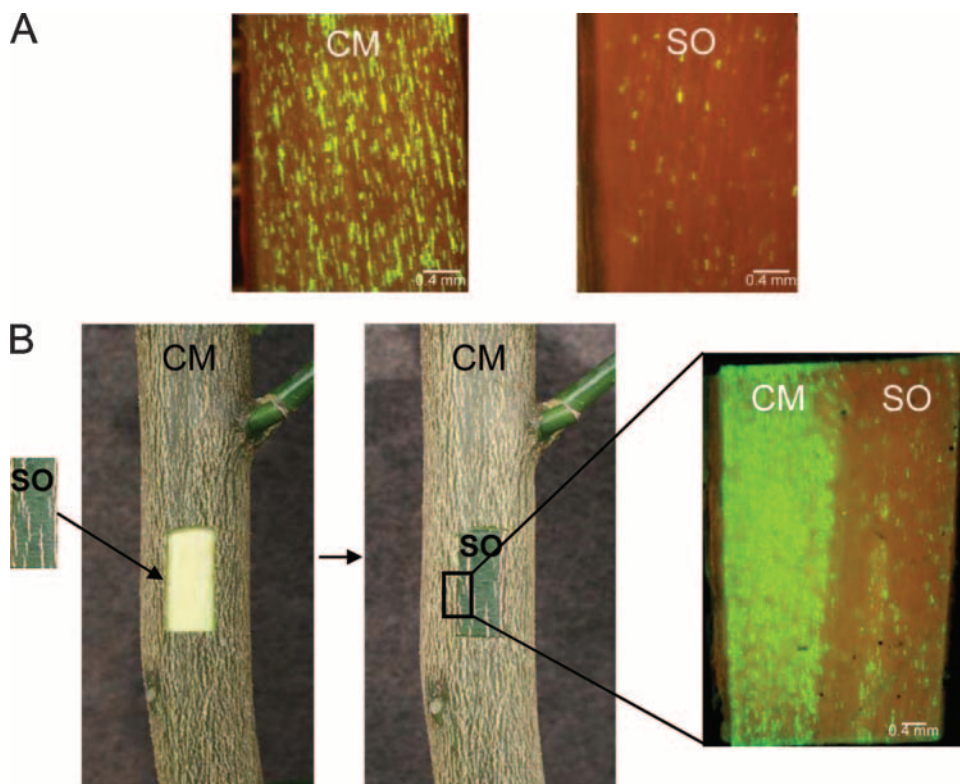


FIG. 5. (A) Detection of GFP fluorescence in phloem-associated cells on the internal surface of bark of *C. macrophylla* and sour orange shoots from a “duplex” plant created by grafting of sour orange onto a *C. macrophylla* tree infected with CTV-BC5/GFP. The image was taken at 6 months after the graft was done. (B) Scheme of the “bark patch” experiment. A small piece of bark was excised from the stem of a *C. macrophylla* tree and replaced with a bark piece of an equal size from a sour orange tree. The substituted bark patch was then allowed to become grafted in place. Six months after inoculation with GFP-expressing CTV, fluorescence was observed in the bark tissue excised from *C. macrophylla* at a region containing grafted bark patch of sour orange. CM, *C. macrophylla*; SO, sour orange.

*phylla* suggest that the long-distance movement mechanism is reduced in sour orange. However, even in the more susceptible host the long-distance movement mechanism of CTV appears to be inefficient, since the vast majority of phloem-associated cells were not infected. Additionally, there appears to be a fixed limit of cell-to-cell movement in each host. Cell-to-cell movement was limited to 12 cells or less in *C. macrophylla* and Mexican lime and essentially did not occur in sour orange and grapefruit. However, even in *C. macrophylla*, there was variation in how far the virus could move, resulting in clusters of different sizes, demonstrating that the limitation was not due to a particular cell type.

The observed movement and distribution of CTV correspond with observations of aphid transmissibility from and to specific citrus species. It has been observed that grapefruit and sour orange are poor donor hosts for aphids to acquire the virus. The reduced infection sites and the single cells would be expected to be smaller targets for aphid stylets or to provide reduced amounts of virus in sieve elements for aphid feeding. Similarly, these species are poorer receptor hosts for aphid transmission experiments. Apparently, the more limited infection sites correspond with less efficient infection when inoculated by an aphid.

The ability of a virus to systemically invade a plant is due to multiple processes: the ability of the virus to efficiently replicate in a host; the facility of interaction of virus encoded

movement protein(s) with complementary host factors; the ability of the virus to suppress the host RNA silencing surveillance mechanism. Based on the amount of GFP fluorescence, the levels of accumulation of CTV per infected cell in the different hosts did not appear to differ substantially. Thus, the major cause of the differences of virus titers in different hosts appears to be differences in numbers of cells infected, which reflect differences in the effectiveness of movement. It is possible that the movement proteins of CTV interact more efficiently with components of some hosts than others. We have not been able to identify which CTV proteins are involved in movement, because of technical problems associated with only being able to inoculate citrus trees with CTV using intact virions (39). However, based on work with another closterovirus, *Beet yellow virus* (BYV), several proteins were found to be associated with movement. Those include both minor and major coat proteins, p6, HSP70 homolog, and p64 (the ortholog of CTV p61), which are required for cell-to-cell movement (3, 30), and the leader proteinase and p20 protein (that is unique to BYV), which play roles in long-distance transport (29, 31). We assume that similar CTV proteins are involved in movement. Additionally, we also have found that some CTV-specific proteins are required for movement in certain hosts. The p33, p18, and p13 genes of CTV can be deleted with normal infection and movement in *C. macrophylla* (42). However, these genes are needed for virus movement in sour orange and

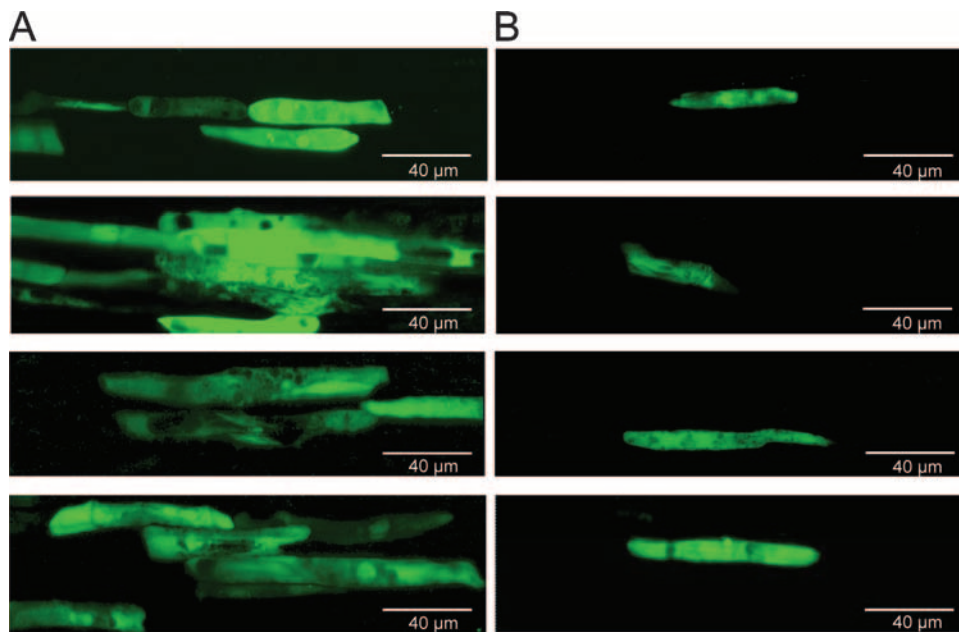


FIG. 6. Confocal laser scanning microscope images of individual infection foci in the bark of several *C. macrophylla* (A) or sour orange (B) plants at 6 weeks after inoculation with CTV-BC5/GFP. The four panels represent replicas from different plants.

grapefruit (T. Satyanarayana et al., unpublished). Perhaps the increased complexity of the movement machinery by the requirement for additional proteins needed for specific movement-related functions in sour orange and grapefruit reduces the efficiency of the long-distance movement in those hosts.

Another possible limitation of CTV movement is inefficient

suppression of RNA silencing. It has been shown that increased suppression of RNA silencing of phloem-limited viruses leads to more extensive virus spread (36, 46). One possibility is that in *C. macrophylla*, RNA silencing is suppressed enough for infection of a few cells in a cluster, but not enough for further spread, and in sour orange, there is even less sup-

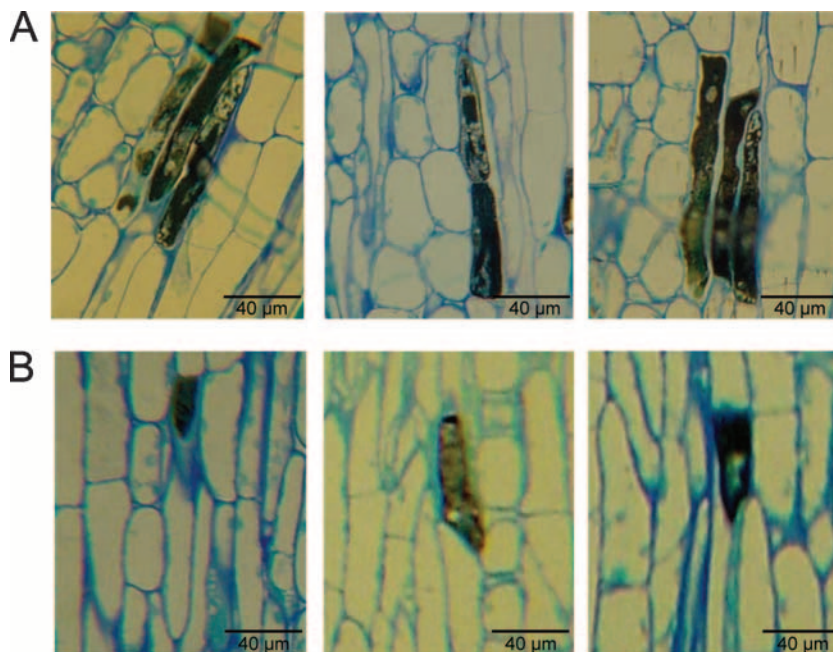


FIG. 7. Light microscope images of infection foci in the bark of *C. macrophylla* (A) or sour orange (B) infected with wild-type CTV at 6 weeks after inoculation. Tissue sections of fixed samples from infected trees were labeled with CTV-specific antibodies as primary antibodies and antibodies with attached 10-nm gold particles as secondary antibodies. To visualize infected cells at the light microscopy level, labeling was further enhanced with the silver enhancement technique.

pression. Though CTV has three different genes that have been identified as RNA silencing suppressors (25), none of them is able to alleviate phloem limitation of the virus, and they could possibly have different functional modes in various citrus species.

An important question relative to the survival of a virus in a plant is what is the source of inoculum for infection of new growth of the plant. Since CTV often is found in single cells or small clusters of cells, a first question is what is the time period that an infected cell serves as a source of inoculum for newly developing cells? For how long is the virus exported from a cell after it becomes infected? In well-examined systems like TMV in tobacco, the movement machinery is thought to coincide with replication. Thus, in tobacco infected with TMV, the cell would be expected to be a source of inoculum for other cells for only 3 to 4 days. It is difficult to determine whether the infected cell could serve as a movement-driven inoculum source at later times, because the surrounding destinations have already been used. However, with CTV in citrus trees it is not known whether the infected cells serve as a source of inoculum only during the replication process or whether virus is exported from the cell for the movement and spread of the infection long after replication has stopped in that cell. In *C. macrophylla* the virus is able to move cell to cell, resulting in clusters. Even though the cell-to-cell spread was limited to 3 to 12 cells in the newly infected flushes, there was some evidence that some cell-to-cell spread continued as the tree grew. As the tree trunk grows, the phloem grows horizontally. When we examined bark pieces from trunks of older trees that have been infected for 1 to 3 years, we still found strong GFP fluorescence. From our experience with TMV-GFP in tobacco, GFP fluorescence fades in a couple of weeks, suggesting that bright fluorescence is an indication of recent replication of CTV. This result suggests that as the more susceptible tree grows, CTV is able to continue cell-to-cell spread horizontally into newly developing phloem-associated cells. This continued replication could serve as a nearby inoculum source for new flushes at the ends of the tree limbs. The question about inoculum source and virus survival in a plant appears to be particularly interesting for sour orange, where the virus is localized to individual cells and apparently is not able to move cell to cell. Further elucidation of this process is necessary to understand how CTV establishes and maintains systemic infections in this and other less-susceptible citrus hosts.

Overall, results of this work emphasize that there is a continuum of different degrees of systemic invasion of plants by viruses based on the ratios of cell-to-cell and long-distance movement. In high-titer viruses that invade most of the cells of the plant, like TMV in tobacco, both long-distance and cell-to-cell movement are efficient. As we move along the continuum to phloem-limited viruses, the process of long-distance movement may continue but cell-to-cell movement becomes more limited, confining the virus to cells surrounding sieve elements. Movement of CTV in citrus is even more limited. CTV in the susceptible hosts *C. macrophylla* and Mexican lime exhibited both limited long-distance and limited cell-to-cell movement. Infection in sour orange appeared to approach the extreme, with even more limited long-distance movement and no cell-to-cell movement. So, to generalize, at one extreme of the continuum of different degrees of systemic invasion by

viruses based on ratios of cell-to-cell and long-distance movement is sour orange, with limited long-distance movement and essentially no cell-to-cell movement. An example of the other extreme of the continuum is the *Citrus leprosis virus*, which has no long-distance movement and moves cell to cell only a few millimeters within localized chlorotic spots (24). This virus causes a serious disease because it is transmitted efficiently by so many mites that essentially the whole tree becomes infected. Yet, the complete lack of one movement mechanism in the cases of these viruses does not result in resistance. These viruses at both extremes of the continuum survive in these hosts in nature.

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