Tomato Infectious Chlorosis Virus Has a Bipartite Genome and Induces Phloem-Limited Inclusions Characteristic of the Closteroviruses


First, second, and fourth authors: USDA/ARS, U.S. Agricultural Research Station, 1636 E. Atisal St., Salinas, CA 93905; and third and fifth authors: Department of Plant Pathology, University of California, Davis 95616.

The mention of firm names or trade products does not imply endorsement or recommendation by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Accepted for publication 4 March 1996.

ABSTRACT


Tomato infectious chlorosis virus (TICV) is a newly described closterovirus. Virions purified from TICV-infected plants contained two single-stranded (ss) RNAs, one of approximately 7,800 (RNA 1) and the other 7,400 (RNA 2) nucleotides. Double-stranded (ds) RNA analysis showed two prominent dsRNAs of approximately 7,800 and 7,400 bp, as well as several smaller dsRNAs. The TICV virion ssRNAs were used for cDNA cloning. Of 200 cDNA clones analyzed, 10 clones containing cDNAs ranging in size from about 900 to 1,500 nucleotides were used to generate digoxigenin-UTP-labeled transcripts. These transcripts hybridized with the TICV ssRNAs in Northern blot hybridization analyses and were used in dot-blot analyses to confirm TICV infection in several host plants including tomato, potato, Physalis wrightii, Nicotiana clevelandii, and artichoke. None of the probes reacted with any uninfected host plant tested or with plants infected with four other clostero- or closter-like viruses including lettuce infectious yellows closterovirus, lettuce chlorosis virus, cucurbit yellow stunting disorder virus, and beet pseudo yellow virus. Northern blot hybridization analyses using selected riboprobes showed no detectable homology between TICV dsRNA 1 and 2, or between subsets of smaller dsRNAs. Inclusion bodies, characteristic of the closteroviruses, were consistently associated with the phloem of TICV-infected N. clevelandii.

Additional keywords: greenhouse whitefly, Trialeurodes vaporariorum.

Tomato infectious chlorosis virus (TICV) was first described by Duffus et al. (9,10) when it was found infecting tomato plants in Orange, San Diego, San Benito, and Yolo counties of California. Symptoms on tomato plants include interveinal yellowing, necrosis, and brittleness of leaves, accompanied by severe yield losses. TICV is transmitted by the greenhouse whitefly, Trialeurodes vaporariorum (Westwood) in a semipersistent manner. The host range of TICV includes several important agronomic crop plants including tomato (Lycopersicon esculentum Miller), tomatillo (Physalis ixocarpa Brot.), potato (Solanum tuberosum L.), artichoke (Cynara scolymus L.), petunia (Petunia hybridra Vilm.), and lettuce (Lactuca sativa L.). Extracts from infected plants and purified virion preparations contain threadlike, flexuous, filamentous particles with a modal length of 850 to 900 nm and a width of 12 nm. This newly described virus is tentatively considered to belong to the genus Closterovirus, based on particle morphology, symptoms on infected plants, and vector specificity (9,10).

Closterovirus taxonomy is rapidly evolving. The International Committee on the Taxonomy of Viruses (ICTV) currently recognizes only six viruses as definitive members of this group, as well as several tentative members (2). Some viruses that were previously proposed as possible closteroviruses on the basis of their flexuous filamentous virions (5,13,24) have been moved to the genera Capillovirus or Trichovirus (3,26). These viruses are reportedly not phloem-limited and do not produce vesicles (5,7,13,24). The definitive closteroviruses with long (1,200 to 2,000 nm) particles are reported to be phloem-limited and produce characteristic cytoplasmic vesicles (7,11,12). Aphids are the vectors for the definitive closteroviruses; although there is a subgroup of clostero- or closter-like viruses including lettuce infectious yellows virus (LIYV) (22,23), lettuce chlorosis virus (LCV) (30), beet pseudo yellows virus (BPYV) (2,7), cucurbit yellow stunting disorder virus (CYSVD) (15), sweet potato sunken vein virus (SPSV) (6,29), and TICV that are transmitted by whiteflies. Only two of these closteroviruses, BPYV and TICV, are reported to be transmitted by the greenhouse whitefly, T. vaporariorum. However, TICV is distinct from BPYV based on serological differences between their coat proteins, retention time in the vector (BPYV is retained longer than TICV), and host range (tomato is not a host for BPYV and members of the Cucurbitaceae are not hosts of TICV) (9,10).

All closteroviruses, except two reported in the literature so far, LIYV (22,23) and TICV (this report), have a single genomic single-stranded (ss) RNA. The genome of LIYV has two ssRNAs corresponding to 8,118 and 7,193 nucleotides. When aligned with the citrus tristeza closterovirus (CTV) and beet yellows closterovirus (BYV) genomes, LIYV appears to have a similar gene organization, but in a divided genome (7,20,23).

This study reports the results from partial cDNA cloning and hybridization analysis of TICV ssRNA and double-stranded (ds) RNAs. cRNA probes were used to verify the host range of this virus and were evaluated for rapid screening of plant samples. Light microscopy of virus inclusions was used to determine the cellular location of TICV. The information generated from this study should help to better understand the relationship between TICV and other closteroviruses.
MATERIALS AND METHODS

RNA isolation and analysis. TICV was propagated in, and virions were purified from, Nicotiana clevelandii. Gray plants as described by Duffus et al. (10). Purified virion preparations (400 μg) were lyophilized in a 10% sucrose solution at a 1:1 ratio (vol/vol). The virion RNAs were extracted by resuspension in 356 μl of diethylpyrocarbonate (DEPC)-treated water, 2 μl of Proteinase K (20 mg/ml), 40 μl of 10% sodium dodecyl sulfate (SDS), and 2 μl of RNasin (40 units/μl, Promega Corp., Madison, WI). After 30 min of incubation at 37°C, the solution was extracted twice using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. After precipitation, the RNA was solubilized in DEPC-treated water, denatured in glyoxal and dimethyl sulfoxide (DMSO) (25), and analyzed on a 1% agarose gel in Loen’s buffer (0.07 M Trizma base, 2 mM EDTA, 0.07 M Na2HPO4 [pH 7.8]). The 0.24- to 9.5-kb RNA ladder (BRL, Grand Island, NY) was used for size standards.

cDNA cloning and Northern blot hybridization analysis. Freshly prepared virion RNA (4 μg) was polyadenylated according to Huijet et al. (19) and used as the template for cDNA synthesis. Subsequent steps were performed according to the Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (BRL). The MAX Efficiency DH5α Competent Cells (BRL) were used for transformation and propagation of the recombinant plasmids. Colonies were selected and plasmids were prepared according to the rapid boiling method of Holmes and Quigley (18). The sizes of the cloned cDNAs were determined by digestion of recombinant plasmids with MluI followed by electrophoresis in a 1% agarose gel in TAE. Ten cDNA clones, ranging from approximately 0.9 to 1.5 kb, were used to generate digoxigenin (DIG)-UTP-labeled transcripts using the DIG-Labeling and Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

TICV ssRNAs and dsRNAs were analyzed by gel electrophoresis. After staining with ethidium bromide (0.5 μg/ml), agarose gels were soaked in 50 mM NaOH for 10 min followed by neutralization in 20X SSC (3 M sodium chloride, 0.3 M sodium citrate [pH 7.0]) for 20 min. RNAs were transferred to positively charged nylon membranes (Boehringer Mannheim Biochemicals) overnight using an Optiblot Transfer Unit (Scotlab, Shelton, CT). Membranes were washed for 2 min in 2X SSC and UV-crosslinked (120,000 μJ/cm2) using a Hoefer UVC 500 Crosslinker (Hoefer Scientific Instruments, San Francisco, CA). Hybridizations were performed at 55°C overnight. Washes, incubation with anti-DIG alkaline phosphatase, and development with Lumi-Phos 500 (Boehringer Mannheim Biochemicals) were performed according to manufacturer’s instructions. Chemiluminescence was detected on X-ray film.

Dot-blot analysis. Plant tissue samples were prepared for dot-blot analysis according to Hadidi and Yang (14). One gram of tissue was triturated to a fine powder in a mortar containing liquid nitrogen. Three milliliters of extraction buffer (0.1 M glycine-NaOH, 50 mM NaCl, 1 mM EDTA, 2% SDS, 1% sodium lauryl sarcosine [pH 9.0]) was added to the samples followed by vigorous shaking for 20 min. Samples were extracted with an equal volume of phenol/chloroform (1:1) and shaken again for 20 min. Samples were centrifuged at 5,000 × g for 20 min, the aqueous phase was recovered, and nucleic acids were precipitated with ethanol. The nucleic acid pellets were solubilized in 400 μl TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). One-microliter samples were applied to nylon membranes, air-dried, UV-crosslinked, and used for hybridization analyses as described above.

Light microscopy. Infected and noninoculated N. clevelandii tissues were sectioned by hand and stained with Azure A accord-

ing to Christie and Edwardson (4) and viewed with a Standard 25 Zeiss microscope (Carl Zeiss, Inc., Oberkochen, Germany).

RESULTS

RNA isolation and analysis. Two ssRNAs, one approximately 7.8 and the other 7.4 kb, were resolved by electrophoresis of TICV virion RNA in a denaturing agarose gel (Fig. 1). The mobilities of the two RNAs were similar and were best resolved by electrophoresis at 50 V for at least 4 h with frequent buffer circulation. The larger of the two RNAs (RNA 1) stained less intensely than the smaller RNA (RNA 2).

When dsRNAs were initially analyzed in nondenaturing agarose gels by electrophoresis for less than 4 h at 50 V, only a single prominent dsRNA of about 7.8 kb and several less intensely staining dsRNAs were resolved (data not shown). However, when dsRNAs were analyzed after electrophoresis for 12 to 16 h at 50 V, two prominent dsRNAs were consistently resolved as well as several smaller dsRNAs (Fig. 2A). The two large dsRNAs had estimated sizes of 7.8 and 7.4 kb based on relative mobilities compared with tobacco mosaic virus (TMV) and tomato bushy stunt virus (TBSV) dsRNAs. Five smaller dsRNAs were also detected by ethidium bromide staining. Their sizes were estimated to be 5.3, 5.0, 4.2, 3.8, and 3.4 kb, respectively. No dsRNAs were present in similar extracts from uninfected N. clevelandii tissue (data not shown).

cDNA cloning and Northern blot hybridization analysis. Two hundred recombinant plasmids were isolated and screened for cDNA inserts. The cloned cDNAs ranged from about 300 bp to 4,000 bp; the majority being between 300 and 1,000 bp. Ten recombinant plasmids were used to generate specific DIG-labeled transcripts for Northern blot and dot-blot hybridization analyses. When virion RNAs were used for hybridization analyses, all probes tested hybridized with virion RNAs, but it was not possible to determine whether the hybridizations were to RNA 1 or RNA 2. Therefore, dsRNAs were used to determine the specificity of hybridization for the labeled transcripts. Specific hybridizations of selected labeled transcripts with RNA 1 or RNA 2 were best resolved using dsRNA electrophoresed for 16 h at 50 V in a 1.5% agarose gel (Fig. 2A and B). Two clones each were selected that hybridized specifically with either dsRNA 1 (pSPT7-19 and pSPT7-46) or dsRNA 2 (pSPT4-26 and pSPT4-16). In addition,

Fig. 1. Ethidium bromide-stained 1% agarose gel of extracted and denatured tomato infectious chlorosis virus (TICV) virion single-stranded (ss) RNAs. Electrophoresis was for 5 h at 50 V constant. Lane 1, ssRNA extracted from purified virions of TICV. The approximate sizes of RNA 1 (7.8 kb) and RNA 2 (7.4 kb) are indicated on the left. Lane 2, Sizes of RNA standards are indicated on the right.

Vol. 86, No. 6, 1996 623
each RNA-specific probe tested hybridized with a different subset of minor dsRNAs that corresponded to those identified by ethidium bromide-stained gels (Fig. 2A and B). The results from these Northern hybridization analyses indicated that the cDNA clones used in these experiments corresponded to two groups of nonhomologous TICV RNAs.

**Dot-blot analysis.** All 10 probes reacted in repeated tests with nucleic acid extracts from plants that were inoculated with TICV by *T. vaporariorum* and showed symptoms of TICV infection. These included *N. clevelandii*, *Physalis wrightii*, artichoke, potato, and tomato plants. No hybridization reactions were observed with extracts from corresponding uninfected plants. Likewise, no reactions were observed with extracts from *N. clevelandii* plants infected with LCV, BPYV, or LIYV, or with extracts from cucumbers (*Cucumis sativus* L.) plants infected with CYSDV, or with extracts from uninfected cucumber plants (Fig. 3).

**Light microscopy.** Cytoplasmic inclusions similar to those induced by other phloem-limited closteroviruses (4) were consistently observed in phloem tissues of TICV-infected, but not healthy, plants and were obvious even under low magnifications (Fig. 4A). Inclusions stained purple to red-violet with Azure A and consisted of fibrous aggregates that often appeared as large vacuolate inclusions in cells (Fig. 4B). Often, red-violet, densely stained plugs were also observed in these same phloem tissues (Fig. 4C). Phloem tissue from uninfected *N. clevelandii* did not stain with Azure A (Fig. 4D).

**DISCUSSION**

The data presented here provide additional evidence for the incorporation of TICV into the genus *Closterovirus*. TICV appears to be more similar to LIYV than to the remaining members of the

genus, in that it has a bipartite genome. The difference in electrophoretic mobility between the two RNAs of TICV is less than that between the two RNAs of LIYV and is, therefore, more difficult to resolve by agarose gel electrophoresis. However, with extended electrophoresis, two RNAs are clearly resolved. These TICV RNAs have been shown to be nonhomologous based on the specific hybridization of each major, and subsets of minor, dsRNAs with separate, labeled cRNA probes.

Historically, the main criterion for identification of a closterovirus was the structure and appearance of the virus particles. According to earlier reviews (5,7), the short particle closteroviruses were neither associated with the phloem nor produced cytoplasmic vesicles. Some of these viruses have been reassigned to either the genera *Trichovirus* or *Capillovirus*. However, members of the bipartite closterovirus subgroup also have short particles, ranging from approximately 750 to 900 nm in length. For example, measurements of over 250 virus particles from leaf discs of LIYV-infected *N. clevelandii* showed the normal particle length to be about 750 to 800 nm (H.-Y. Liu, unpublished data), in contrast to previous reports of 1,800 nm in which only the longest virus particles were recorded (8). This virus is associated with the phloem and produces vesicles as well (17). Likewise, particles of the whitefly-transmitted closterovirus SPSVV are reported to be 850 to 950 nm in length, and SPSVV also occurs in the phloem and produces vesicles (6,29). Although the SPSVV genome was initially reported to have a single ssRNA, recent studies (R. I. Hamilton and J. B. Vetten, personal communications) indicate it to be bipartite. TICV also appears to belong to the subgroup of bicomponent closteroviruses because it i) has a modal length of 850 to 900 nm; ii) is associated with the phloem as seen with light microscopy of infected tissue, and iii) is transmitted by whiteflies. Electron microscopy studies are currently underway to determine the possible formation of cytoplasmic vesicles like those of LIYV and SPSVV.

The hybridization of RNA 2-specific TICV clones with at least five smaller TICV dsRNAs may be an indication of the presence of several subgenomic (sg) RNAs, as suggested for CTV (16) and for LIYV (22). The presence of several small dsRNAs observed in the ethidium bromide-stained gel was further supported by the corresponding Northern hybridization analysis. Whereas RNA 1-

---

Fig. 2. Ethidium bromide-stained 1.5% agarose gel and Northern blot hybridization analysis of tomato infectious chlorosis virus (TICV) double-stranded (ds) RNAs. Electrophoresis was for 16 h at 60 V, and subsequent Northern blot hybridization analysis showed two distinct major TICV dsRNAs and several additional minor ones. A, Arrows to the left of ethidium bromide-stained gel indicate the upper two major dsRNAs and at least five minor dsRNAs. B, Northern blot hybridization analysis of replicate dsRNA samples probed with lane 1, pSPT7-46; lane 2, pSPT7-19; lane 3, pSPT4-26; and lane 4, pSPT4-16. Arrows to the left of the Northern blot corresponded to dsRNA 1 and one minor dsRNA that hybridized with pSPT7-46 and pSPT7-19 (lanes 1 and 2). Arrows to the right corresponded to dsRNA 2 and five minor dsRNAs that hybridize with pSPT4-26 and pSPT4-16 (lanes 3 and 4).

Fig. 3. Dot-blot analysis of nucleic acid extracts from plant tissue probed with a selected cRNA clone (pSPT4-16) of tomato infectious chlorosis virus (TICV). Column I contains extracts from samples infected with TICV: A, TICV in tomato; B, TICV in potato; C, TICV in *Physalis wrightii*; D, TICV in *Nicotiana clevelandii*; and E, TICV in artichoke. Column 2 contains extracts from corresponding uninfected plants: A, tomato; B, potato; C, *P. wrightii*; D, *N. clevelandii*; and E, artichoke. Column 3 contains plant extracts for other closteroviruses and corresponding uninfected cucumber: A, beet pseudo yellow virus (BPYV)-infected *N. clevelandii*; B, lettuce infectious yellows virus (LIYV)-infected *N. clevelandii*; C, lettuce chlorosis virus (LCV)-infected *N. clevelandii*; D, cucumber yellow stunt disorder (CYSDV)-infected cucumber; and E, uninfected cucumber.
specific clones hybridized with only one small dsRNA, RNA 2-
specific clones hybridized with at least five. In addition, the eth-
idium bromide-stained gel showed additional small dsRNAs other
than those that were detected in the Northern analysis, and none of
these were present in healthy plant tissue extracts. If the genome
organization of TICV was similar to that of LIYV in which sev-
eral unique genes characteristic of the closteroviruses were located
on RNA 2 (i.e., HSP70 homologue, coat protein, divergent coat
protein), then a larger number of open reading frames that were
expressed via sgRNAs would also be expected with TICV RNA 2
rather than with RNA 1.

The widespread nature of TICV in tomato in California, the host
range that includes several important agronomic crops, and the
transmission by *T. vaporariorum* will likely necessitate a rapid
and accurate indexing technique for field- and greenhouse-grown
plant samples. The dot-blot analysis appears to be sensitive and
highly specific, with no background observed for any healthy host
plant tested or for four other closteroviruses. All 10 nonradioac-
tive probes tested reacted similarly in dot-blots with extracts of
the infected hosts tested. However, reactions of nucleic acid ex-
tracts from TICV-infected artichoke were consistently weaker than
reactions with the solanaceous hosts tested.

It appears that a new subgroup of closteroviruses is being re-
ognized with the recent descriptions of whitefly-transmitted, bi-
partite members of this group including LIYV (2), and possibly
TICV and SPSVV. The term “biclovirus” (for bipartite closterovi-
ruses) has been suggested to represent the LIYV-related members
of the genus *Closterovirus* (7). The definitive classification of

---

**Fig. 4.** Freehand sections from tomato infectious chlorosis virus (TICV)-infected and noninoculated *Nicotiana clevelandii* tissue stained with Azure A and photographed with a Zeiss standard microscope. **A,** Sections through TICV-infected *N. clevelandii* crown tissue photographed at 10X magnification. Note red-violet staining of phloem tissue (P). **B,** TICV-infected tissue photographed at 100X showing nucleus (N) and vacuolate virus inclusions (I) throughout phloem cell. (V = vacuolate inclusion.) **C,** Infected tissue photographed at 100X showing densely staining pluglike inclusions (I) in the phloem cell. Bar represents 0.1 μ in A and 1 μ in B. **D,** Noninoculated *N. clevelandii* tissue stained with Azure A, photographed at 10X magnification.
TICV as a bipartite closterovirus, however, should await sequence information and the identification of the HSP70 gene (1,21,27), as reported for other closteroviruses. Further work on TICV is in progress to more fully understand its relationship to other closteroviruses and to improve rapid and sensitive detection techniques for routine diagnostic assays.

LITERATURE CITED