Comparison of Diagnostic Techniques for Detecting Tomato Infectious Chlorosis Virus

R. H. Li, G. C. Wisler, H.-Y. Liu, and J. E. Duffus, U.S. Department of Agriculture, Agricultural Research Service, 1636 East Alisal Street, Salinas, CA 93905

ABSTRACT

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A polyclonal antiserum prepared against purified virions of tomato infectious chlorosis virus (TICV) was used to evaluate serological tests for its detection, to determine its distribution in infected plants, to study relationships among isolates of this virus, and to detect it in field samples. A cRNA probe representing TICV RNA 1 and RNA 2 was used in dot blot hybridization tests. A reverse transcriptase-polymerase chain reaction (RT-PCR) assay was also developed for detection of TICV isolates. The comparative study of these four techniques indicated that RT-PCR was 100-fold more sensitive than enzyme-linked immunosorbent assay (ELISA), Western blot, and dot blot hybridization assays for TICV detection. TICV was detected in leaf, stem, flower, and root tissues of the infected tomato plants. However, the virus was not uniformly distributed throughout the infected tomato plants, and the highest viral concentration was observed in fully developed young tomato leaves at the onset of yellowing symptoms. The virus was detected by indirect ELISA, Western blot, dot blot hybridization, and RT-PCR assays in laboratory-infected tomato, tomatillo, potato, and *Nicotiana clevelandii* and in naturally infected tomato, petunia, and *Ranunculus* sp. plants obtained from commercial sources. These tests indicate that there are apparently no detectable serological or nucleic acid differences among four TICV isolates obtained from Orange and Yolo Counties of California or from North Carolina or Italy.

Tomato infectious chlorosis virus (TICV) is a recently recognized pathogen of tomato (7). The virus is distributed in greenhouses throughout California and infects several important crops and ornamental plants, including tomato (*Lycopersicon esculentum* L.), tomatillo (*Physalis ixocarpa* Brot.), potato (*Solanum tuberosum* L.), artichoke (*Cynara scolymus* L.), lettuce (*Lactuca sativa* L.), and petunia (*Petunia × hybrida* Vilm.). Symptoms on tomato plants include interveinal yellowing, necrosis, and brittleness of leaves accompanied by severe yield loss. TICV is transmitted in a semipersistent manner by the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood).

TICV belongs to a growing group of bipartite, whitefly-transmitted closteroviruses, which includes lettuce infectious yellows virus (LIYV) (13) and sweet potato sunken vein virus (11). TICV has long, flexuous, filamentous particles that measure about 850 to 900 × 12 nm (7). The

virus induces cytoplasmic vesicles characteristic of closteroviruses in the phloem of infected plants (18). TICV has a bipartite, single-stranded RNA genome, and its genomic RNA 1 and RNA 2 are 7.8 and 7.4 kb in length, respectively.

Unlike those of most plant viral diseases, the yellowing symptoms caused by TICV are not easily recognized because they are mainly on older leaves and resemble those caused by malnutrition or senescence. The mild symptoms of TICV, its phloem-limited nature, and its low concentration in infected plants make diagnosis difficult in its natural hosts. Routine detection of the virus in indicator plants by vector transmission is not only tedious, often requiring several weeks to obtain results, but impractical for large numbers of samples. Because of the problems with detection and the need for more reliable and rapid systems to screen for the presence of TICV, we have investigated the potential use of protein- and nucleic acid-based assays for its detection. A polyclonal antiserum against purified TICV virions is available for serological detection of the virus (7). Dot blot hybridization with digoxigenin (DIG)-labeled TICV cRNA probes has been developed and used for detecting TICV in laboratory-inoculated plants such as *Nicotiana clevelandii* Gray, *Physalis wrightii* Gray, and tomato (18). Reverse transcriptase-polymerase chain reaction (RT-PCR)-based assays have been frequently used for detection of plant RNA

viruses and generally have been found to be more sensitive than any of the previously used detection assays (10,12). The cloning and sequencing of the 3'-end portion of RNA 1 and RNA 2 of the TICV-O isolate allowed us to design primers for RT-PCR detection of the virus (*unpublished*). We report here the development and application of the enzyme-linked immunosorbent assay (ELISA), Western blot, dot blot hybridization, and RT-PCR procedures for TICV detection and their relative merits as diagnostic procedures. These methods greatly increase the speed and ease of detecting TICV in infected plants and may be useful in studying TICV. A preliminary report of this study has been published (14).

MATERIALS AND METHODS

Virus sources. Five TICV isolates from tomato were used in this study. TICV-Orange (TICV-O) was obtained from commercial tomato plants in the Irvine area of Orange County, California, during the fall of 1993. TICV-Davis (TICV-D) was obtained from tomato plants grown at the University of California, Davis, in Yolo County, California. TICV-SB was obtained from tomato plants grown in a commercial greenhouse in San Benito County, California. TICV-North Carolina (TICV-NC) was supplied by P. S. Shoemaker, North Carolina State University. TICV-Italy (TICV-I) was supplied by P. Caciagli, Institute di Fitovirologia Applicata, Torino, Italy. Tomato chlorosis virus (ToCV), another whitefly-transmitted closterovirus that infects tomatoes, was obtained from greenhouse-grown tomato plants collected in Florida (17). All TICV and ToCV isolates were maintained in tomato and *N. clevelandii* plants and transferred from plant to plant via inoculation with greenhouse whiteflies. Beet yellows virus (BYV) was obtained from sugar beet plants grown in breeding plots in Salinas, California. BYV was maintained in sugar beet plants and transferred to *Chenopodium capitatum* (L.) Asch. via inoculation with green peach aphids, *Myzus persicae* (Sulzer). LIYV was obtained from commercial lettuce plants collected in the Imperial Valley of California during the fall of 1981 (5). Lettuce chlorosis virus (LCV) was obtained from commercial lettuce plants collected in the Imperial Valley of California during 1991 (8). Both LIYV and LCV were maintained in *C. murale* L. and transferred

Corresponding author: H.-Y. Liu;
telephone: 408/755-2826; fax: 408/753-2866;
E-mail: hslingliu@asrr.arsusda.gov

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from plant to plant via inoculation with sweetpotato whiteflies, *Bemisia tabaci* (Gennadius). Cucurbit yellow stunting disorder virus (CYSDV) (9) was obtained from Israel in 1993 (6). CYSDV was maintained in melon (*Cucumis melo* L.) and transferred from plant to plant via inoculation with sweetpotato whiteflies. Beet pseudo-yellows virus (BPYV) was obtained from field-infected dandelion plants collected in the Salinas Valley, California (16). BPYV was maintained in cucumber (*Cucumis sativus* L.) and transferred from plant to plant via inoculation with greenhouse whiteflies. Citrus tristeza virus (CTV) antigen was provided by S. M. Garnsey of the U.S. Department of Agriculture, Agricultural Research Service, Orlando, Florida.

Serological assays. Indirect ELISA was conducted by first coating plates with antigens prepared from infected and healthy leaf tissues in 10 volumes of coating buffer (0.05 M sodium carbonate, pH 9.6). TICV antiserum (1:1,000) was cross-absorbed with healthy sap of tomato and *N. clelandii* leaves (1:10, wt/vol, in phosphate-buffered saline [PBS]) and added to the plates. Goat anti-rabbit immunoglobulin IgG (whole molecule) alkaline phosphatase conjugate (Sigma, St. Louis, MO) was added to the plates and incubated. Plates were washed four times with PBST (0.5%

Tween 20 in PBS) between each step. A_{405} values were measured 2 h after addition of the substrate (*p*-nitrophenyl phosphate) at room temperature.

Virus was detected by Western blot assays as previously described with minor modification (15). Proteins extracted from plant leaves were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with Prestained Protein Molecular Weight Standards (Bethesda Research Laboratories [BRL] Life Technologies, Gaithersburg, MD). The proteins were transferred to a nitrocellulose membrane and detected with TICV IgG purified from the healthy plant sap cross-absorbed antiserum (3).

Nucleic acid-based assays. Dot blot hybridization assays were made by using DIG-labeled cRNA probes. Two recombinant plasmid clones, pSPT8 and pSPT6, were used for cRNA probe synthesis. The 4.0-kb pSPT8 and the 2.0-kb pSPT6 were complementary to the 3'-end region of RNA 1 and RNA 2, respectively. DIG-labeled RNAs were transcribed from linearized pSPT8 and pSPT6 with SP6 polymerase according to the instructions of the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN). Total nucleic acid extracts were isolated from leaf tissues of infected and healthy plants according to the following method de-

scribed by de Vries et al. (4) with modifications. Leaf tissue (0.4 g) was ground in liquid nitrogen into a fine powder with a mortar and pestle. Two volumes (0.8 ml, wt/vol) of RNA extracting buffer (0.1 M Tris-HCl, pH 8.0, containing 0.1 M LiCl, 10 mM EDTA, and 1% SDS) and two volumes (0.8 ml) of phenol were added and

Table 1. Comparison of tomato infectious chlorosis virus (TICV) isolates and other closteroviruses by indirect ELISA with TICV antiserum

Antigen ^a	TICV antiserum ^b (A_{405}) ^c
TICV-O	1.187
TICV-D	0.724
TICV-NC	0.572
TICV-I	1.513
ToCV	0.059
Healthy tomato	0.006
BYV	0.014
LIYV	0.012
LCV	0.019
Healthy <i>Chenopodium murale</i>	0.019
CYSDV	0.024
Healthy cucumber	0.034

^a TICV-O, Orange County isolate; TICV-D, Davis isolate; TICV-NC, North Carolina isolate; TICV-I, Italy isolate; ToCV, tomato chlorosis virus; BYV, beet yellows virus; LIYV, lettuce infectious yellows virus; LCV, lettuce chlorosis virus; and CYSDV, cucurbit yellow stunting disorder virus.

^b TICV antiserum is a polyclonal antiserum against purified TICV-O virions. The antiserum was cross-absorbed with healthy tomato and *Nicotiana clelandii* saps.

^c Absorbances were taken 2 h after addition of substrate and represent averages of eight wells.

Fig. 1. Western blot analysis of four different isolates of tomato infectious chlorosis virus (TICV) in tomato and five other closteroviruses: beet yellows virus (BYV) in *Chenopodium capitatum*, lettuce infectious yellows virus (LIYV) and lettuce chlorosis virus (LCV) in *C. murale*, cucurbit yellow stunting disorder virus (CYSDV) in melon, and beet pseudo-yellows virus (BPYV) in cucumber. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and reacted with TICV immunoglobulin G, which was purified from cross-absorbed antiserum. Healthy lanes refer to corresponding noninoculated plants. Prestained protein molecular weight standards are in the far left lane.

Fig. 2. Detection of isolates of tomato infectious chlorosis virus (TICV) by dot blot hybridization. The total nucleic acid samples extracted from both infected and healthy plants were spotted on a nylon membrane and hybridized with a TICV probe containing digoxigenin-labeled cRNA preparations from plasmid clones representing both RNA 1 and RNA 2 of the virus. Closteroviruses tomato chlorosis virus (ToCV), beet yellows virus (BYV), lettuce infectious yellows virus (LIYV), lettuce chlorosis virus (LCV), and cucurbit yellow stunting disorder virus (CYSDV) were used to confirm probe specificity. Specific hosts tested correspond to those in Figure 1.

homogenized briefly. Homogenates were heated at 90°C for 5 min, and supernatants were extracted with phenol, chloroform, and isoamyl alcohol (25:24:1). Total nucleic acids were recovered by precipitation with isopropanol and dissolved in 80 µl of sterile water. Two-microliter samples or their serial dilutions were spotted onto nylon membranes, air dried, UV-cross-linked, and used for hybridization assays as described by Wisler et al. (18).

RT-PCR. For RT-PCR assays, two oligonucleotide primers were designed based on the 3'-end region sequence of TICV RNA 2 and synthesized by the DNA Synthesis Core Laboratory at the University of Florida's Interdisciplinary Center of Biotechnology Research. The upstream primer I (5'-ATGAGGTCTTTTCACAGTGG-3') is identical to nucleotide sequence 264 to 282 nt from start codon in an open reading frame (ORF) at the 3'-end region of RNA 2, which is an analog of the ORF 6 of LIYV (13). The downstream primer II (5'-GTCCGAACTGATTGAACC-3') is complementary to nucleotide sequence 959 to 940 nt before the stop codon of the same ORF. The predicted length of the amplified fragment is approximately 700 bp. Primers were diluted to about 100 ng/µl. Single-stranded cDNA was synthesized from 2 µl of total nucleic acids extracted from plant leaf tissue by using Superscript II RNase H⁻ reverse transcriptase (BRL), according to the manufacturer's instructions. One microliter of primer II was used in a total volume of 20 µl. For PCR, the 5-µl RT mixture was added to a tube containing 100 ng of each primer, 0.2 µM of each dNTP (deoxynucleoside triphosphate), 3 µl of 25 mM MgCl₂, 5 µl of 10× reaction buffer, and 1 U of *Taq* DNA polymerase

(Promega, Madison, WI) in a total of 50 µl of reaction volume. The reaction was run at the following temperature-cycling profile (touchdown PCR): 18 cycles of 1.25 min at 94°C, 1.5 min at 72 to 55°C (temperature decline of 1°C per cycle), and 2 min at 72°C; 17 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C followed by a 10-min extension at 72°C. PCR products were separated by electrophoresis on 1% agarose gel (BRL) in TBE (45 mM Tris-borate and 1 mM EDTA), stained with ethidium bromide, and photographed.

After electrophoresis, PCR products were transferred for Southern hybridization to positively charged nylon membranes with an Optiblot Transfer Unit (Scotlab, Shelton, CT) overnight in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Membranes were washed for 5 min in 2× SSC and UV-crosslinked (240,000 µJ/cm²) with a Hoefer UVC 500 Crosslinker (Hoefer Scientific Instruments, San Francisco, CA). Hybridizations were performed at 55°C, and the bound probes were detected with the DIG luminescent detection kit (Boehringer Mannheim) according to the manufacturer's instructions. Results were recorded on Kodak X-Omat film.

TICV distribution in tomato plants. ELISA, Western blot, dot blot, and RT-PCR assays were compared to evaluate their sensitivity and reliability in detecting TICV in infected plants. Western blot assay was chosen to assess variation in viral concentrations within individual plants and to detect TICV in yellowing plants collected from commercial greenhouses. Samples were collected from different tissues (leaf, stem, flower, and root) and from each leaf of several tomato and *N. cleve-*

landii plants infected with TICV-O, TICV-D, and TICV-I isolates and prepared for Western blot assay.

RESULTS

TICV detection by serological assays.

Repeated tests in which nonabsorbed polyclonal antiserum was used against TICV in indirect ELISA resulted in high background readings with the healthy control. However, after cross-absorption, TICV antiserum was suitable for serological detection in indirect ELISA (Table 1). TICV antiserum reacted with all four TICV isolates, and it also reacted weakly with ToCV but not with other closteroviruses, including BYV, LIYV, LCV, BPYV, and CYSDV. In other ELISA tests, TICV antiserum did not react with other closteroviruses, such as CTV and BPYV (*data not shown*).

The specific serological reactivity of TICV isolates with homologous antiserum was tested further by Western blot assays (Fig. 1). All four TICV isolates tested were easily detected, as the TICV coat protein of 32 kDa was observed. Such serological reactivity was not observed when BYV, LIYV, LCV, CYSDV, and healthy tissues were used. In other Western blots, ToCV, another whitefly-transmitted tomato closterovirus, reacted only weakly against TICV IgG (*data not shown*).

TICV detection by nucleic acid-based assays.

DIG-labeled cRNA probes prepared from two plasmid clones, pSPT8 and pSPT6, were used in dot blot hybridization assays. It has been determined that pSPT8 represents the 3' end half of RNA 1 and

Fig. 3. Detection of isolates of tomato infectious chlorosis virus (TICV) by reverse transcriptase-polymerase chain reaction. Primers I and II were used to amplify a DNA fragment of approximately 700 bp from cDNAs synthesized with primer II from total nucleic acid samples extracted from infected and healthy plants. No visible products were observed in healthy tomato or in five other closteroviruses: tomato chlorosis virus (ToCV), beet yellows virus (BYV), lettuce infectious yellows virus (LIYV), lettuce chlorosis virus (LCV), or cucurbit yellow stunting disorder virus (CYSDV). DNA 1-kb ladder was in the far left lane. (A) Ethidium bromide-stained gel. (B) Southern blot after hybridization with a TICV probe containing digoxigenin-labeled cRNA preparations from plasmid clones representing both RNA 1 and RNA 2 of the virus.

Fig. 4. Sensitivity comparisons of enzyme-linked immunosorbent assay (ELISA), Western blot, dot blot hybridization, and reverse transcriptase-polymerase chain reaction (RT-PCR) assays for detection of tomato infectious chlorosis virus (TICV). Tenfold dilutions of the TICV-infected *Nicotiana cleveandii* leaf tissue samples corresponding to 10 mg, 1 mg, 100 µg, 10 µg, 1 µg, and 100 ng of leaf tissue were used. The mean absorbance values in ELISA were averages of eight wells. The coat protein band in the Western blot was 32 kDa. The RT-PCR assay amplified a DNA fragment of approximately 700 bp from a TICV-infected plant.

pSPT6 the 3' end one-quarter of RNA 2, accounting for about 40% of TICV genome (*unpublished data*). All five TICV isolates were readily detected from total nucleic acids extracted from infected plants (Fig. 2). Hybridization with ToCV, BYV, LIYV, LCV, CYSDV, and healthy controls was not observed.

When total nucleic acid preparations from infected plants were used as templates for RT-PCR, a single DNA product of the predicted length of approximately 700 bp was observed in agarose gels for all five TICV isolates (Fig. 3A). No PCR products were obtained with ToCV, BYV, LIYV, LCV, CYSDV, and healthy controls. Southern hybridization verified the viral origin of the amplified product (Fig. 3B). Preliminary tests in which 35 cycles were used with the theoretical annealing temperature of 55°C resulted in visible nonspecific amplification products on the gels. The use of the touchdown PCR profile eliminated the amplification of nonspecific products.

The sensitivity of ELISA, Western blot, dot blot hybridization, and RT-PCR assays was directly compared by using a dilution series of samples for TICV detection in infected plants. Leaf tissue from a TICV-infected *N. clevelandii* plant was processed for virus detection by all four assays. As shown in Figure 4, the virus was detected at dilutions equivalent to 100, 10, and 1 µg and 100 ng of leaf tissue in ELISA, Western blot, dot blot hybridization, and RT-PCR, respectively.

Virus could be detected from tomato plants inoculated with TICV isolates by whitefly transmission in the laboratory. In our sensitivity tests, the infected samples were ELISA positive in dilutions up to 1:1,000 (Fig. 4). However, the ELISA results of samples collected from naturally infected plants were not always consistent with bioassays, possibly because of low virus concentration in old plants. Although the Western blot assay is not as sensitive as RT-PCR, it was used in our study to detect TICV in infected plants, since it is reliable and convenient. TICV could be detected by Western blots in leaf, stem, flower, and root tissues of infected tomato plants (Fig. 5); however, the relative concentration of TICV within infected plants varied. The virus concentration was higher in fully de-

veloped young leaves with visible yellowing symptoms than in older leaves with severe yellowing symptoms (Fig. 6). The virus concentration in old or new leaves was either low or undetectable. These patterns of the virus distribution were found in tomato and *N. clevelandii* plants infected by TICV-O, TICV-D, and TICV-I isolates.

The Western blot assay was also used to detect TICV in plants collected from commercial greenhouses (Table 2). The virus was detected in 23 samples from 108 tomato plants, in two samples from 16 petunia plants, and in all three ranunculus plants collected from commercial greenhouses in California, Florida, and North Carolina. All TICV-positive samples were from

Fig. 5. Distribution of tomato infectious chlorosis virus (TICV) in different tissues of an infected tomato plant determined by Western blot. Protein samples from leaf, stem, root, and flower tissues of the TICV-infected tomato plant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and reacted with TICV immunoglobulin G, which was purified from cross-absorbed antiserum. Healthy lanes refer to samples from a noninoculated plant. Prestained protein molecular weight standards are on the far left.

Fig. 6. Distribution of tomato infectious chlorosis virus (TICV) in different leaves of an infected tomato plant determined by Western blot. Protein extracts from different leaves of a TICV-infected tomato plant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and reacted with TICV immunoglobulin G, which was purified from cross-absorbed antiserum. The number above each lane is leaf age, oldest (1st) to youngest (10th). Healthy lane refers to leaf sample from a noninoculated plant. Prestained protein molecular weight standards are on the far left.

Table 2. Detection of tomato infectious chlorosis virus (TICV) by Western blot in plants grown in commercial greenhouses^a

Host	Positive samples/ total samples
Tomato	23/108
Petunia	2/16
Ranunculus	3/3

^a The cross-absorbed TICV immunoglobulin G was used in the Western blots for TICV detection. The positive samples were determined as the TICV coat protein of 32 kDa was observed.

California and North Carolina, while ToCV was the causal agent for yellowed tomatoes collected from Florida (17).

DISCUSSION

Four methods for the detection of TICV have been evaluated. The results of ELISA and Western blot assays indicate that TICV polyclonal antiserum against the purified virions is suitable for TICV detection in infected plants. Cross-reactions with healthy plant antigens in ELISA and Western blot assays are not surprising, because of the mild nature of the purification procedure used to obtain the virus preparations (7). However, these reactions were eliminated by cross-absorption of the TICV antiserum with healthy tomato and *N. clevelandii* saps. The cross-absorbed TICV antiserum reacted strongly with all TICV isolates but not with most other closteroviruses tested, except ToCV. A weak serological reaction was detected with ToCV when the TICV antiserum was used in both ELISA and Western blot assays. This supports the fact that there are only limited serological relationships among the closteroviruses (1,2). The results also indicate that there is no detectable serological difference among TICV isolates. There was no significant difference in A_{405} readings for four isolates in ELISA, and the protein banding patterns of these isolates were identical in the Western blots.

The result of dot blot hybridization shows that the DIG-labeled cRNA probe prepared from two clones representing the 3'-end regions of RNA 1 and RNA 2 is sensitive and specific for TICV detection. The probe could detect TICV in a 10- μ g equivalent of leaf tissue, which was similar to the sensitivity of the Western blot assay. The probe hybridized only with the extracts of TICV-infected plants and not with those of other closteroviruses tested, including ToCV, and of healthy plants. TICV isolates can also be detected from total nucleic acid extracts of infected plants by RT-PCR under the conditions described. The primer pair I and II, which amplifies a 700-bp fragment, is specific for TICV detection. The 700-bp DNA fragment was observed only in samples of TICV-infected plants. This fragment was absent from the samples of other closteroviruses tested and from healthy plants. Consistent with the serological data, the results of the nucleic acid-based assays, dot blot hybridization, and RT-PCR indicate that there is no detectable difference among the TICV isolates.

As expected, RT-PCR is the most sensitive assay of the four tested and could be used to confirm samples that are inconclusive in ELISA, Western blot, and dot blot hybridization assays. For routine tests of large numbers of samples, the Western blot and dot blot hybridization assays are more convenient. Furthermore, the similar sensi-

tivity of the Western blot and dot blot hybridization assays makes the Western blot assay the preferred preliminary test for TICV detection because of its speed and ease.

Analysis of different tissues or leaves of infected plants by Western blots reveals that the distribution of TICV within plants is uneven. The virus concentration detected in the fully developed young leaves with mild yellowing symptoms was higher than that in old leaf, stem, flower, and root tissues. Once the antiserum is cross-absorbed, no reactions are observed with healthy plant tissues. Distribution of TICV also differed among leaves in infected tomato and *N. clevelandii* plants, where higher virus concentration was observed in the fully developed young leaves (leaves three to five) than in both older and younger leaves. Therefore, when samples are taken for TICV detection, the fully developed young leaves of an infected plant should be selected for the most accurate detection of the virus.

TICV has been obtained from yellowing tomato plants grown in greenhouses or in fields in different locations of California since the virus was first found in southern California in 1993. The TICV host range includes several vegetable and ornamental plants besides tomato, and the virus may be maintained in greenhouse plants and spread from one location to another by shipment of these plants. The presence of the virus in ornamental flower plants from commercial nurseries in California and in tomato samples collected from North Carolina supports this, since germ plasm exchange between the two states may have occurred. The presence of the virus in Italy indicates that the TICV distribution might be worldwide.

When TICV antiserum was used to study the yellowing tomato samples collected from Florida, only very weak reactions were obtained in both ELISA and Western blot assays. Further studies with dot blot hybridization and RT-PCR revealed that ToCV, a new whitefly-transmitted closterovirus, is the causal agent of the disease. The sensitivity and rapidity of the techniques described here support their application as valuable tools in the study of the etiology and epidemiology of TICV and related closteroviruses.

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