Reverse-Transcription Polymerase Chain Reaction Detection of Citrus Tristeza Virus in Aphids

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ABSTRACT

Mehta, P., Brlansky, R. H., Gowda, S., and Yokomi, R. K. 1997. Reverse-transcription polymerase chain reaction detection of citrus tristeza virus in aphids. *Plant Dis.* 81:1066-1069.

A rapid and simple reverse-transcription polymerase chain reaction (RT-PCR) method was developed for the detection of citrus tristeza virus (CTV) in three aphid species. Seven CTV isolates from a worldwide isolate collection were used for aphid acquisition feeding by three aphid species. These included the most efficient CTV vector, the brown citrus aphid, *Toxoptera citricida*; the melon aphid, *Aphis gossypii*; and the green peach aphid, *Myzus persicae*, a non-vector for CTV. A short procedure for nucleic acid extraction from single or groups of aphids was developed. Nucleic acid extracts from 1, 3, 5, and 10 aphids with acquisition-access periods of 24 and 48 h were reverse transcribed and amplified using primers for the coat protein gene of the Florida B3 (T-36) isolate of CTV. PCR-amplified fragments of approximately 670 bp were obtained from all the isolates tested and the amplified product from the aphids fed on citrus infected with isolate B3 was confirmed as the CTV coat protein gene by digesting with various restriction enzymes. This technique will be useful in investigations of CTV-vector-plant interactions and CTV epidemiology.

Additional keywords: acquisition, semi-persistent

Citrus tristeza virus (CTV) is an economically important pathogen of citrus in many production areas of the world. It causes two important diseases—a decline of citrus on sour orange (*C. aurantium* L.) rootstock (CTV-decline) and stem pitting of many cultivars regardless of rootstock. The virus is a phloem-limited closterovirus, consisting of 11 nm × 2,000 nm flexuous particles and has a positive sense single-stranded RNA genome of 19,296 nt, which makes it the largest known plant virus genome (2,15). CTV is vectored by aphids in a noncirculative semipersistent manner (16). Of the aphid species listed as vectors, the most studied ones are *Toxoptera citricida* (Kirkaldy), *Aphis gossypii* (Glover), and *Aphis spiraecola* Patch (1). Of these, *T. citricida*, the brown citrus aphid (BrCA), is the most important and efficient CTV vector (5,13). Single *T. citricida* have been reported to be 80–100% efficient in transmitting many CTV isolates (17). *A. gossypii* has also been shown to be an efficient vector for some CTV isolates (1). Hosts for the BrCA are plant species in the Rutaceae and include most *Citrus*

spp., hybrids, and other citrus relatives (6). Tristeza problems, either CTV-decline or stem pitting of orange or grapefruit (*C. paradisi* Macf.), have generally occurred whenever BrCA has appeared (12).

Recently, BrCA was discovered in the continental U.S. in Florida (8). Because field isolates of CTV are a complex of various strains, studies on the transmission of endemic and exotic isolates of CTV by aphid vectors are important to better understand the nature of these isolates (or strains) (3). To facilitate investigations of CTV-BrCA-plant interactions, we needed to develop a rapid method for the detection of CTV in single and up to 10 aphids. This report describes the development of a short procedure for nucleic acid extraction from single or groups of aphids and detection of CTV using reverse-transcription polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Virus isolates and aphid species. Seven CTV isolates from the CTV exotic isolate collection at the USDA Beltsville Agricultural Research Center, Beltsville, MD were used in this study. These were B3, B6, B14, B151, B152, B165, and B199. Their geographic origin and indicator symptoms (based on the standard host range [7]) are listed in Table 1.

All CTV isolates were graft inoculated or aphid transmitted into Madam vinous sweet orange (*Citrus sinensis* [L.] Osbeck) plants and these infected plants were used for virus acquisition by aphids. The B3

isolate also was graft inoculated into Kaffir lime or combava (*C. hystrix* Hort. ex Tan.).

Aphid species used for virus acquisition were two vectors, *T. citricida* and *A. gossypii*, and a nonvector species, *Myzus persicae* (Sulzer). Colonies of *T. citricida* were maintained on healthy Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*) seedlings, *A. gossypii* were reared on okra (*Hibiscus esculentus*), and *M. persicae* were reared on 'California Wonder' pepper (*Capsicum annum* L.) and cabbage (*Brassica oleracea* L. var. *capitata*).

Acquisition of CTV by vector and non-vector aphid species. Aphids from each vector species, *T. citricida* and *A. gossypii*, and from the non-vector species, *M. persicae*, were placed on CTV-infected citrus plants in separate aphid-proof cages. After acquisition-access periods (AAP) of 24 and 48 h, aphids were collected using a fine camel hair brush and stored at –20°C. Aphids fed on healthy citrus plants as controls also were collected and stored as described.

Nucleic acid extraction from aphids and citrus tissues. A short extraction procedure was designed. One, 3, 5, and 10 aphids were placed in sterile microfuge tubes; 25, 50, 75, and 100 µl of cold extraction buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added, respectively; and the mixture was ground thoroughly with a plastic pestle. Extracts from 100 mg of the petiole and the bark tissues from healthy citrus were prepared by grinding the materials to a powder in liquid nitrogen in a sterile mortar and pestle to which 1 ml of extraction buffer was added. Immediately after homogenization, the solutions were vortexed briefly and centrifuged at 15,000 rpm for 5 min, and the supernatant was collected in sterile microfuge tubes. Twenty, 40, 60, and 80 µl of the supernatant from 1, 3, 5, and 10 aphids, respectively, and 200 µl from the citrus tissue extracts were transferred to sterile microfuge tubes and 10, 20, 30, and 40 µl of the Gene Releaser (Bio Ventures, Inc., TN) in case of aphids and 50 µl in case of leaf tissues were added, respectively. The solutions were mixed and heated for 20 min at 75°C and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was collected into sterile microfuge tubes, and 0.1 vol of 3 M sodium acetate and 2.5 vol of 95% ethanol were added. The tubes were inverted to mix the contents and were incubated for 30 min at –70°C. After incuba-

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Florida Agricultural Experiment Station Journal Series No. R-05268.

Accepted for publication 10 June 1997.

Publication no. D-1997-0703-02R

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tion, the tubes were centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was discarded. The pellet was washed with 500 µl of 70% ethanol, dried for 10 min under vacuum, resuspended in 20 µl of sterile distilled water, and stored at -20°C.

Fig. 1. Polymerase chain reaction (PCR)-amplified product of B-3 (T-36) isolate of citrus tristeza virus (CTV) from individual and multiple viruliferous *Toxoptera citricida*. Amplification of approximately 670-bp fragments with the primer pair C100 and C74. An aliquot (5 µl) of the 50 µl PCR mixture was analyzed in a 1% agarose gel. Lane 1, 1-kb ladder size marker (75 bp-12 kb, Gibco BRL); lanes 2, 4, 6, and 8, PCR-amplified product from viruliferous 10, 5, 3, and single *T. citricida*, respectively, with an acquisition-access period of 48 h; lanes 3, 5, 7, and 9, PCR-amplified product from viruliferous 10, 5, 3, and 1 *T. citricida*, respectively, with an acquisition-access period of 24 h; lanes 10 and 11, PCR-amplified product from individual nonviruliferous *T. citricida* and healthy citrus bark tissue, respectively; lane 12, PCR-amplified CTV-DNA fragment from the plasmid containing the CTV (B3) 3' region including the CP gene.

Fig. 2. Restriction digestion analysis of polymerase chain reaction (PCR)-amplified fragments of B3 (T-36) isolate of CTV from individual *Toxoptera citricida*. Lane 1, 1-kb ladder size marker (75 bp-12 kb, Gibco BRL, NY); lanes 2 and 3, undigested CTV-DNA from cloned CTV CP gene and PCR-amplified fragment from individual *T. citricida*, respectively; lanes 4 and 5, *BstEII* digestion of cloned CTV CP and from individual *T. citricida*, respectively; lanes 6 and 7, *ClaI* digestion of cloned CTV CP fragment and from individual *T. citricida*, respectively.

Reverse transcription. The first strand cDNA was synthesized using the nucleic acid extracted from the aphid species as templates and C74 as the primer. The total reaction volume was 25 µl, which contained 15 units of AMV reverse transcriptase (Promega, Madison, WI), 25 units of rRNasin (Promega, Madison, WI), and 0.24 mM each of dATP, dCTP, dGTP, dTTP (Idaho Technology, Inc., ID). The contents were mixed gently and incubated at 42°C for 60 min.

PCR amplification. The primers C100 (GGCGGAATTCGACGACGAAACAAA GAAA, viral sense polarity with an *EcoRI* site at the 5' end) and C74 (GAAGATCT-TCAACGTGTGTTGAATTTC, complementary sense polarity with *BglII* site at the 5' end) (unpublished) were used to amplify the coat protein (CP) gene of CTV. The primers C100 and C74 were designed based on the nucleotide sequence for the CP gene of isolate B3 with unique restriction sites at the ends to facilitate further cloning and amplification of a 670-bp CP gene product from the plasmid containing the CP gene. The amplification was per-

formed in a final volume of 50 µl containing 10 µl of cDNA; 5 units of *Taq* DNA polymerase (Promega, Madison, WI); 2.5 mM $MgCl_2$; 0.12 mM each of dATP, dCTP, dGTP, and dTTP (Idaho Technology Inc., ID); and 0.3 µM of each primer. PCR was performed in a programmable thermal controller (model PTC-100, MJ Research Inc., Watertown, MA) using the following parameters: one cycle at 94°C for 2 min; 35 cycles at 94°C for 5 s (denaturation), 48°C for 10 s (annealing), and 72°C for 10 s (primer extension); followed by one cycle at 72°C for 2 min.

PCR-amplified fragments from the aphids containing the B3 isolate of CTV were digested with *BstEII* and *ClaI* (New England Biolabs, MA). The digested fragments and nondigested fragments were electrophoresed in 1% agarose gels, respectively, in Tris-acetate-EDTA buffer and detected using UV light after staining in ethidium bromide.

RESULTS

Detection of CTV in single and multiple viruliferous *T. citricida* by RT-PCR.

Fig. 3. Polymerase chain reaction (PCR)-amplified product of various isolates of citrus tristeza virus (CTV) from a group of single and multiple *T. citricida*. Amplification of approximately 670-bp fragments with the primer pair C100 and C74. An aliquot (5 µl) of the 50 µl PCR mixture was analyzed in a 1% agarose gel for all isolates except for isolate B199. Lane 1, 1-kb ladder size marker (75 bp-12 kb, Gibco BRL, NY); lanes 2-13, a group of three and single *T. citricida* with CTV isolates B14 (lanes 2, 3), B165 (lanes 4, 5), B6 (lanes 6, 7), B199 (lanes 8, 9), B151 (lanes 10, 11), B152 (lanes 12, 13), respectively; lanes 14 and 15, no PCR-amplified products from a group of three and single nonviruliferous *T. citricida*; lane 16, no amplified product from healthy citrus bark tissue and lane 17, the amplified product from the plasmid positive control.

Table 1. Origin and biological properties of the citrus tristeza virus isolates used for virus acquisition by aphid species

Virus isolate	Geographic origin	Disease caused ^a	Indicator symptoms ^b
B3	Florida	CD	MoSY, MoVC
B6	California	CD, OSP, GSP	SvSY, SvVC
B14	Brazil	CD, OSP, GSP	SvSY, SvVC
B151	California	SSO	MVC
B152	California	CD, GSP	SSY, MoVc
B165	India	CD, OSP, GSP	SSY, SVC
B199	Israel	CD, GSP, OSP	MoSY, MoVC

^a CD = citrus decline, OSP = orange (*Citrus sinensis* [L.] Osbeck) stem pitting, GSP = grapefruit (*C. paradisi* Macf.) stem pitting, SSO = stunting in sweet orange on sour orange (*C. aurantium* L.).

^b MSY = mild seedling yellows in Eureka lemon (*C. limon* [L.] Burm f.) or sour orange (*C. aurantium* L.), MoSY = moderate seedling yellows, SSY = strong seedling yellows, SvSY = severe seedling yellows, MVC = mild vein clearing in Mexican lime (*C. aurantifolia* [Christm.] Swing.), MoVC = moderate vein clearing in Mexican lime, SVC = strong vein clearing in Mexican lime, SvVC = severe vein clearing in Mexican lime.

RT-PCR-amplified fragments of approximately 670 bp were obtained using C100 and C74 primers with the devised nucleic acid extraction procedure (Fig. 1). Amplified fragments corresponding in size to the CTV-CP gene were obtained from 10, 5, 3, and single *T. citricida* fed on B3-infected plants for AAP of 48 and 24 h, respectively. No DNA fragments were obtained either from healthy citrus leaves or non-viruliferous *T. citricida*.

Restriction digestion analysis. Restriction digests of RT-PCR-amplified fragments from single *T. citricida* fed on the B-3 isolate of CTV yielded fragments with both of the restriction enzymes *Bst*EII and *Cl*al (Fig. 2). The sizes of these fragments agreed with predicted values (384 and 286 bp) for *Bst*EII and (571 and 99 bp) for *Cl*al estimated from the sequence of the CP gene of CTV (11,15), indicating that *T. citricida* acquired CTV during the acquisition-access period on infected citrus.

Detection of various CTV isolates in single and multiple viruliferous *T. citricida* by RT-PCR. Six different CTV isolates designated as B14, B165, B6, B199, B151, and B152 were detected from single and groups of three *T. citricida* by RT-PCR (Fig. 3). The intensity of the bands were similar for all isolates except B199. In order to obtain the detection of B199 shown in Fig. 3, 10 µl of PCR-amplified product was loaded in the agarose gel as compared to 5 µl for the other isolates. No differences were observed in detection from three or single aphids (Fig.3). DNA fragments were not amplified either from

healthy citrus tissues or nonviruliferous *T. citricida*.

Detection of CTV in *T. citricida*, *A. gossypii* and *M. persicae* by RT-PCR. RT-PCR-amplified fragments of approximately 670 bp corresponding to the CP gene were obtained from single and groups of three *T. citricida*, *A. gossypii*, and *M. persicae* fed on B3 isolate of CTV for an AAP of 48 h (Fig. 4). The intensity of the bands obtained from *A. gossypii* and *M. persicae* were similar to those obtained with *T. citricida*, the most efficient CTV vector. No DNA fragments were obtained either from healthy citrus leaves or nonviruliferous *T. citricida*, *A. gossypii*, and *M. persicae*.

DISCUSSION

RT-PCR was successfully used to detect CTV in single and groups of 3, 5, and 10 aphids. This method detected CTV in three aphid species—*T. citricida*, *A. gossypii*, and *M. persicae*. No bands were obtained from either healthy citrus tissues or non-viruliferous aphids.

The described procedure for nucleic acid extraction is simple, efficient, and can be used on a large number of samples in a short period of time. Because RT-PCR amplifies nucleic acids, it is useful in overcoming many difficulties encountered with serological methods, such as low antigen titer, availability of antibodies, and cross reactivity of antibodies with heterologous antigens. RT-PCR is faster than methods such as squash blot and dot blot hybridization and is not based on the use of radioisotopes such as ³²P. The sensitivity of the method shown here in detecting CTV in single as well as multiple aphids should allow the processing of composite samples of aphid vectors.

Enzyme-linked immunosorbent assay has been used to detect CTV in plants and 11 aphid species, including many nonvectors of CTV (4). Similarly, we found that CTV was detected in a nonvector, *M. persicae*. Apparently, CTV is acquired by different species of aphids independent of their ability to transmit it. The exact location of semipersistently transmitted closteroviruses like CTV in aphids is unknown. Semipersistent, noncirculative transmission is characterized by an increasing transmission probability with an increase in acquisition period and a retention period of several days (10). This suggests that virus can accumulate in the vector and resist dissociation. Since infectivity is not retained through a molt, the virus retention site is likely the cuticle or intima that lines the foregut. RT-PCR could be useful in determining if virus is located in dissected mouthparts of vector and nonvector aphids. If both vector and nonvectors contain virus in mouthparts, then only the ability to have virus released is the difference between vectors and nonvectors. Immunolabeling coupled with electron microscopy also may help provide more information on virus

location in the aphid. Murant et al. (14) found virus-like particles of anthriscus yellows virus and parsnip yellow fleck virus in a matrix material attached to the intima lining of the pharynx. Attachment of the virus to its vector might be either the consequence of attachment of the viral protein to cell membranes of the lining of the alimentary canal or indirectly by helper factors (9). Understanding the interaction of plant viruses in vectors and nonvectors may lead to novel ways to disrupt acquisition or transmission.

ACKNOWLEDGMENTS

This study was supported by USDA/ARS/IFAS Cooperative Agreement 48-6617-4-108. We thank the USDA, ARS Foreign Disease Science Research Unit, Frederick, MD, for use of the facilities for some of the aphid-CTV acquisition and V. Damsteegt and C. Gouin-Behe for assistance.

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Fig. 4. Polymerase chain reaction (PCR)-amplified product of B3 (T-36) isolate of citrus tristeza virus (CTV) from different aphid species. These aphids were given an acquisition-access period of 48 h on CTV-infected citrus plants. Amplification of approximately 670-bp fragments with the primer pair C100 and C74. An aliquot (5 µl) of the 50 µl PCR mixture was analyzed in a 1% agarose gel. Lane 1, 1-kb ladder size marker (75 bp-12 kb, Gibco, BRL, NY); lanes 2 and 3, PCR-amplified product from groups of three and single *Toxoptera citricida* with B3 isolate of CTV; lanes 5 and 6, PCR-amplified product from groups of three and single *A. gossypii* with B3 isolate of CTV; lanes 8 and 9, PCR-amplified product from groups of three and single *M. persicae* with B3 isolate of CTV; lanes 4, 7, and 10, no amplified product from individual nonviruliferous *T. citricida*, *A. gossypii* and *M. persicae*, respectively; lane 11, no amplified product from healthy citrus bark tissue and lane 12, the PCR-amplified product from the plasmid control.

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