Detection of Citrus Tristeza Virus by Print Capture and Squash Capture-PCR in Plant Tissues and Single Aphids

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ABSTRACT. Two sensitive methods using immobilized targets on membranes (hemi-nested-PCR and nested-PCR in a single closed tube) were applied successfully for amplification of citrus tristeza virus (CTV) targets from plant material (print capture) and aphids (squash capture). Amplification was obtained from different aphid species regardless of transmission efficiency showing that CTV is acquired by different aphid species. The use of preprinted or presquashed targets offers a new and sensitive diagnostic tool for CTV detection for many applications including epidemiological studies.

Index words. Citrus tristeza virus, detection, aphids, *Toxoptera citricida, Aphis gossypii, Aphis nerii, Hyalopterus pruni*, squash capture-PCR, print capture-PCR, hemi-nested-PCR, nested-PCR.

Citrus tristeza virus (CTV) is one of the most detrimental virus diseases of citrus (2), especially where CTV-sensitive rootstocks are used. CTV is vectored by a number of aphid species (Homoptera, Aphididae) in a noncirculative, semi-persistent manner (19). Seven aphid species have been determined as CTV vectors in different geographical areas (13).

Although the transmission efficiency of CTV by *Toxoptera citricida* is high (23), *Aphis gossypii* has also proved to be an efficient vector of the virus in California, Israel and Spain (1, 6, 11, 13). High populations of *A. gossypii* in some Spanish areas were responsible for spread of the tristeza disease (6). The non-efficient vectors, *Toxoptera aurantii* and *Aphis spiraecola* (13), along with uncontrolled movement of infected plant materials, were implicated in the CTV-induced death of about 20 million trees in Spain from 1930-35 to 1989. The recent CTV epidemic from 1989 to present is due to spread by *A. gossypii* and has caused the decline and death of an additional 20 million trees. Other aphids such as *Aphis craccivora, Uroleucon jaceae*, and *Myzus persicae* have been described in India as CTV vectors (20, 21).

The use of certified pathogen-free plants and epidemiological studies to track the incidence and spread of CTV in the field are the two strategies to manage the tristeza problem. Accurate epidemiological data allows the proper recommendation to be given to a grower on what to do to control CTV such as the use of selected varieties grafted on tristeza-tolerant rootstocks produced under a certification scheme.

Sensitive and reliable detection of CTV in plants and vectors is essential for suitable control of the virus and better understanding of the relationships between CTV and its insect vectors. ELISA has been successfully applied for the detection of CTV in plant material (12) and in aphids (7, 8). Nevertheless, in the latter case, positive reactions were obtained only when extracts were prepared from multiple aphids (more than 20). The low titer of semipersistent transmitted viruses in aphids make their serological detection in single individuals difficult.

Recently, detection of CTV targets in a single aphid has been obtained using conventional reverse-transcription polymerase chain reaction (RT-PCR) (14) and a variation called print-, spot-, or squash-cap-

ture PCR (PC- or SC-PCR) (10). These methods use sensitive and simple procedures developed for the preparation of plant and insect samples before PCR amplification without the need of extract preparation (17, 18). This method essentially uses imprinted sections of plant material or squashed aphids on Whatman 3MM paper (10). The immobilized or captured targets are released from the blotted paper with a simple Triton treatment (18).

In this study, the PC- or SC-PCR method, previously applied to other RNA and DNA viruses (9, 10, 15, 17, 18), are coupled to the new techniques of hemi-nested-PCR and nested-PCR in a single closed tube, allowing sensitive amplification of CTV targets from infected plants or aphid tissues directly from the blots on paper without any need of nucleic acid extraction or immunocapture phase.

MATERIAL AND METHODS

Virus sources and plant material preparation. Twenty three characterized Spanish CTV isolates from the IVIA collection, maintained in an insect-proof screenhouse in sweet orange grafted on Troyer citrange in Moncada (Valencia), and the Cuban CTV isolate, Cu50, from the IICF collection maintained in sweet orange in a greenhouse in La Habana, were used in this study. In addition, shoots from sweet orange trees infected with CTV isolate I.7 from CTV epidemiology plots at IVIA, Moncada and naturally-infected trees from the Dominican Republic were used. In addition, 12 Washington Navel sweet orange trees were surveyed (five stems/tree) in Moncada in November. CTV negative controls from different citrus species were kindly provided by Dr. L. Navarro from the pathogen-free germplasm collection maintained at IVIA.

Samples from healthy and CTV-infected plants from the collection or field were collected in individual plastic bags and ground in PBS, pH 7.2 to 7.4, supplemented with 0.2% DIECA (1/20, w/v). Sections of these plant materials were also directly blotted on Whatman 3MM paper (18). Ten imprints from each plant were used (two imprints per stem section) on about 1 cm^2 paper, using both sides of the paper when needed. The printed papers prepared in Valencia were stored at 4°C in plastic bags for as long as 1 mo before being processed. The prints made in Cuba or the Dominican Republic were sent to Valencia by international courier at ambient temperature and stored at 4°C until processed.

To estimate the limits of sensitivity of the print-capture method, an experiment was performed imprinting the paper with a sectioned stem collected from a sweet orange tree infected isolate T-308 in Moncada along with imprints of zero, four, nine, 14 or 19 imprints from a healthy tree.

Preparation of aphids squashes. Aphids collected from field trees or fed on CTV-infected hosts under experimental conditions were individually squashed on Whatman 3MM paper. The round bottom of Eppendorf tubes was used like a pestle to crush each aphid (17).

Seventy-nine *T. citricida* apterae were individually collected and squashed in June 1996 from an established colony on a CTV-infected sweet orange tree in the Dominican Republic. Imprints of sections of shoots from which the aphids were captured were simultaneously tested. Healthy controls were prepared by crushing single *T. citricida* fed on a CTV-free sweet orange tree. The paper membranes were mailed to Valencia at room temperature and stored at 4°C until used. The analysis of the squashed aphids was performed in three different periods: September 1996; April 1997; and April 1998.

Individual *T. citricida* and *A. gossypii* were collected from a colony on

an infected sweet orange tree (isolate Cu50) and a non-infected sweet orange tree in Cuba. The squashed aphids were sent to IVIA by conventional mail and analyzed about 1 mo later.

A. gossypii grown on cotton in a growth chamber were individually transferred to detached young shoots of a CTV infected Washington Navel tree (I.7) maintained in water. The young growing shoots with aphids were caged in 3 × 8 cm plastic cylinders. After a 5 h virus acquisition feeding period, the aphids were collected with a brush, placed on the membrane and squashed. The same operation was performed with *Aphis nerii* and *Hyalopterus pruni*, previously captured in *Nerium oleander* and *Prunus armeniaca*, respectively. Some individuals of each of the three aphid species were fed for 5 h on young shoots of a healthy sweet orange seedling cv. Pineapple as negative controls.

Apterae of *T. citricida* (from Cuba) and *A. gossypii* (from Spain) were fed on sweet orange CTV infected (Cu50 and T-397, respectively) shoots for several acquisition times ranging from 15 min to 48 h. Ten individuals per acquisition period were tested. *T. citricida* were squashed and the blots mailed to IVIA in Valencia; whereas *A. gossypii* were squashed *in situ* and the blots stored at 4°C until used.

Enzyme-linked immunosorbent assay (ELISA). Double antibody sandwich biotin/streptavidin ELISA (4) was performed by coating Nunc Maxisorp immunoplates with 200 µl per well using the commercial kit supplied by INGENASA (Madrid) with a mixture of the CTV-specific monoclonal antibodies (MCAs) 3DF1 and 3CA5 (5).

Immunocapture-PCR (IC-PCR). One hundred µl of clarified plant extract were subjected to immunocapture as previously described (16, 22) using MCAs 3DF1 (0.5 µg/ml) and 3CA5 (0.5 µ/ml) for coating microfuge tubes. Additionally some tubes were coated with plum pox virus (PPV) specific MCA 5B-IVIA (3) at 1 µg/ml, with carbonate buffer alone, and other tubes received no treatment.

Amplification was made by conventional one-step RT-PCR using CTV-specific primers: PT1: 5' GGT TCA CGC ATA CGT TAA GCC TCA CTT and PT2: 5' TAT CAC TAG ACA ATA ACC GGA TGG GTA. Briefly, 25 µl of RT-PCR mix (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3% Triton X100, 1 µM of each primer, 250 µM dNTPs, 0.25U AMV and 0.5U TaqPol) was added to each tube. cDNA synthesis was performed at 42°C for 45 min followed by a denaturation step of 94°C for 2 min. The amplification process consisted of 40 cycles of 92°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. Ten µl of PCR products were analysed by electrophoresis on 3% agarose gels, stained by ethidium bromide and visualized under UV transilluminator. In some cases, the preliminary capture phase of IC-PCR was also used to prepare samples before amplification using the hemi-nested-PCR or nested-PCR in a single closed-tube procedures.

Print and Squash capture preparation of samples before PCR. The method was as previously described (18) and patented (Spanish Patent P9601155). The extraction of the targets from paper harboring either tissue imprints or squashed aphids was performed by using 1% Triton X-100, vortex and incubation (at room temperature). Triton extracts were transferred to RT-PCR tubes and reverse transcription and amplification were done as previously described.

Print and Squash capture-hemi-nested PCR (PC-H-PCR, SC-H-PCR). Hemi-nested PCR was performed in two steps as previously described (17) using extracted targets from paper as indicated above. The first amplification process, consisted of 25 µl of RT-PCR

mix: 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3% Triton X100, 1 µM of primer PA: 5' TAA ACA ACA CAC ACT CTA AGG, 1 µM primer PB: CAT CTG ATT GAA GTG GAC, 250 µM dNTPs, 0.25 U AMV and 0.5 U TaqPol. Reverse transcription was performed at 42°C for 45 min followed by a denaturation step of 94°C for 2 min. The amplification process consisted in 40 cycles of 92°C for 30 sec, 45°C for 30 sec and 72°C for 1 min. After PC/SC-PCR, 1 µl of RT-PCR product was added to a second amplification process. Briefly, the second reaction mix consisted in 10mM Tris-HCl pH8.8, 50mM KCl, 1.5mM $MgCl_2$, 1 µM of primer PB and 1 µM of POD (nested primer): 5' CTT TGG TTC ACG CAT ACG T, 250 µM dNTPs and 0.5U Taq Pol in a total volume of 25 µl. Amplification consisted in 35 cycles of 92°C for 30 sec, 50°C for 30 sec and 72°C for 1 min. Electrophoresis was performed as mentioned above.

Print and Squash capture nested-PCR in a single-closed tube (PC-nested-PCR, SC-nested-PCR). The method was performed using a compartmentalized Eppendorf tube (Spanish Patent P99801642). The end of a standard 200 µl plastic pipette tip introduced into a 0.5-ml PCR tube allows the physical separation of two different PCR cocktails. Extracted targets from paper (see above) were used. After the first round of PCR, the tube was centrifuged and mixture 2 (into the pipette tip) was combined with mixture 1 (into the bottom of the tube). The reaction mix consisted of 30 µl mixture of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 3 mM $MgCl_2$, 250 µM dNTPs, 0.5 µM of external primers (PEX1: 5' TAA ACA ACA CAC ACT CTA AGG and PEX2: 5' CAT CTG ATT GAA GTG GAC), 1.2 U of AMV and 0.6 U of Taq Pol. The reaction mix 2 included a 10 µl mixture of 50 mM KCl, 10 mM Tris-HCl, (pH 9.0), 8 µM of internal primers (PT1: 5' GGT TCA CGC ATA CGT TAA GCC TCA CTT and PT2: 5' TAT CAC TAG ACA ATA ACC GGA TGG GTA). RT-PCR was performed at 42°C for 30 min followed by denaturation at 94°C for 2 min and 20 cycles of amplification (92°C for 40 sec, 45°C for 40 sec and 72°C for 1min 20sec) and nested PCR began with a denaturation phase of 2 min at 94°C followed by 40 cycles of amplification (92°C for 30 sec, 60°C for 30 sec and 72°C for 1 min).

RESULTS

Detection of CTV in plant tissues by different variants of print capture-PCR. The PC-PCR method allowed detection of CTV-immobilized targets on Whatman 3MM paper from all 23 CTV isolates assayed from the IVIA collection. PCR products were of the expected size (131 bp for PC-PCR, 223 bp for PC-H-PCR, and 131 bp for PC-nested-PCR), without the need of extract preparation. The same plants were also found positive for CTV by the conventional IC-PCR using plant extracts.

Twelve field sweet orange trees recently infected by CTV were found positive by DAS-ELISA biotin/streptavidin system and by the different PC-PCR variations assayed. Leaf imprints from infected trees from Cuba and the Dominican Republic were positive by PC-H-PCR and by PC-nested-PCR. No amplification signals were observed from the healthy controls. Positive reactions were obtained from imprinted material which was sent via international courier and stored for 2 yr at 4°C. No differences were observed when compared with other plant material printed and subsequently analyzed.

Analysis of multiple imprints (one from a CTV-infected tree and others from a healthy tree) in the same piece of paper by PC-nested-PCR resulted positive for one, five, 10, and 15 imprints. No reaction

was observed using 20 prints simultaneously. Positive amplifications were obtained independently for the specificity of the coating antibodies. Positive reactions were obtained with 3DF1 and 3CA5 CTV-specific MCAs and the amplified product of 131 bp also was obtained in tubes coated with MCA 5B-IVIA (PPV specific), tubes coated with carbonated buffer or in non-coated tubes.

Detection of CTV targets in single aphids by different variants of Squash capture-PCR. CTV targets from all CTV isolates tested were detected by SC-H-PCR and/or by SC-nested-PCR from individual viruliferous-aphids squashed on paper. No amplification signals were obtained from aphids fed on healthy citrus species.

Seventy-nine *T. citricida* were squashed in June 1996. In September 1996, April 1997 and April 1998 the squashes were analyzed by SC-H-PCR. The results (number of positive/total tested) were:1/15, 4/41 and 1/23, respectively, for the dates of analysis.

T. citricida and *A. gossypii* collected in Cuba on a Cu50-infect sweet orange were squashed and analyzed 1 mo later by SC-nested-PCR. Four of 13 *T. citricida* and four of nine *A. gossypii* gave positive amplifications. No reaction was obtained with non viruliferous aphids used as controls.

The ability of different aphid species to acquire isolate I.7 after 5 h feeding is shown (Table 1) by SC-H-PCR and SC-nested-PCR. CTV targets were amplified in a higher number of aphids by SC-nested-PCR in single closed-tubes than by SC-H-PCR. The aphid species fed on CTV-infected sweet orange showed positive amplifications when assayed. Table 2 shows the results of single aphid tests using SC-nested-PCR after different acquisition periods. After 15 or 30 min of feeding, no positive amplification was obtained from either *T. citricida* or *A. gossypii*. CTV targets were amplified only from a single aphid out of 10 assayed, regardless of the duration of the acquisition period, for the two aphid species.

DISCUSSION

The new RT-PCR variations presented have demonstrated their potential for CTV detection in plant tissues imprinted as well as from aphids squashed on Whatman 3MM paper. Optimization and evaluation of hemi-nested-PCR and nested-PCR were done for amplification of all CTV isolates tested. The primers were designed from the 3' UTR conserved region of CTV genome. The sensitivity of hemi-nested-PCR was higher than conventional RT-PCR (data not shown), as has been demonstrated for other viruses (17). The main disadvantage of hemi-nested-PCR is the need to open the first amplification tube to take 1 µl sample

TABLE 1
AMPLIFICATION OF CITRUS TRISTEZA VIRUS (CTV) TARGETS BY SQUASH CAPTURE (SC) HEMI-NESTED- AND NESTED-PCR FROM APHIDS FED ON DETACHED SHOOTS OF CTV-INFECTED WASHINGTON NAVEL ORANGE

	Detection method[z]	
Aphid species	SC-hemi-nested-PCR	SC-nested-PCR
Aphis gossypii	3/8	36/72
Aphis nerii	2/29	4/19
Hyalopterus pruni	1/19	4/14

[z]Number of aphids with positive CTV amplification/total number of aphids assayed. Aphids were allowed a 5-h acquisition feeding period on shoots infected with CTV isolate I7.

TABLE 2
AMPLIFICATION OF CITRUS TRISTEZA VIRUS TARGETS BY SQUASH NESTED PCR IN A SINGLE CLOSED TUBE FROM *TOXOPTERA CITRICIDA* AND *APHIS GOSSYPII* AFTER DIFFERENT CTV ACQUISITION ACCESS PERIODS

		CTV acquisition access period[z]						
Aphid	CTV isolate	15 min	30 min	60 min	5 h	16 h	24 h	48 h
Toxoptera citricida	Cu50	0	0	2	1	1	1	na
Aphis gossypii	T-397	0	na	1	1	na	1	1

[z]Number of individual aphids out of 10 apterous individuals tested per acquisition period and species in which amplification of CTV targets was positive. na = not analyzed.

as target for the second amplification which is performed in another tube. This operation risks contamination. To avoid this problem, a new method for capture, reverse transcription and nested-PCR in a single closed-tube was developed. The internal primers for nested-PCR were designed from areas of the 3' UTR region having low secondary structure. This gave excellent performance in RT-PCR. Compatible external primers were designed with the lowest annealing temperature whereas internal primers had the highest annealing temperature. This system saves time in the optimization of the reaction because the external primers do not interfere in the second amplification.

The main advantages of the new nested-PCR in a single closed tube is its simplicity, easy establishment of the adequate conditions for the reaction, low risk of contamination, and the high sensitivity of nested-PCR when compared with hemi-nested-PCR.

We also showed the possibility of immobilizing viral targets (by imprinting or by squashing) on membranes. This method opens new possibilities for serological or molecular (hybridization or amplification by PCR) detection (10). The method allows the preparation of the imprints and their long-term storage before analysis by serological or molecular techniques. Up to 15 imprints can be performed on the same piece of paper (1 cm^2) without inhibition of subsequent PCR reactions. The high sensitivity of PC-nested-PCR allows the detection of CTV without the need for a previous capture. Positive amplification was obtained in tubes coated with CTV-specific antibodies as well as in tubes coated with PPV-specific or in uncoated tubes.

Coupled with squash-capture sample preparation (10, 17, 18) the increased sensitivity provided by hemi-nested and nested-PCR allowed the detection of CTV targets in individual wingless aphid of different species without the need of nucleic acid extraction (14). SC-H-PCR and SC-nested-PCR were successfully used to detect CTV in aphid vectors as well as in non-vectors. The acquisition of CTV targets for different aphid species, regardless of their ability to transmit the virus, was demonstrated in 1982 by ELISA (7) and more recently by PCR (14). The data shown in this paper confirms these previous findings.

Increasing the acquisition feeding period did not increase the number of aphids in which CTV targets were amplified. Probably a longer acquisition period increased the viral titer in some aphids instead of increasing the number of viruliferous aphids. SC-nested-PCR could be very useful to verify this hypothesis by performing simultaneous assays of detection and transmission.

The low rate (4.3% to 9.7%) of positive detection of CTV targets in *T. citricida* from the Dominican Republic can be explained by incom-

plete disruption of the squashed aphids. The total disruption and release of CTV target seems to be essential for a good capture on the paper. Nevertheless, this experiment showed that squashed aphids could be stored up to 2 yr before testing. In the experiments with Cuban *T. citricida,* the percentage of CTV-infected aphids collected from the same colony was 30.8%; whereas for *A. gossypii* it was 44.4%. The percentage calculated for Spanish aphids captured in the field: *A. gossypii* (44.4%), *A. nerii* (21.0%) and *H. pruni* (28.6%) after SC-nested-PCR was higher than the percentage calculated for aphids used in experimental assays of acquisition. The percentage of "acquisition-detection" was only 10% for *T. citricida* and *A. gossypii* for acquisition periods of more than 30 min.

Conventional ELISA techniques are sensitive enough to detect CTV in plant material but not in single aphid vectors. Therefore, it was necessary to use highly sensitive techniques like PCR for detection in the vector. However, extract preparation limits the number of samples that can be processed, increases risk of contamination, and involves the release of inhibitors of plant or aphid origin. In addition, procedures for virus detection in a single aphid already described are laborious and require viral nucleic acids extraction. Print and squash capture-PCR offer a number of advantages overcoming these problems. Tissue imprints and squashed aphids can be performed in field conditions, stored and mailed for analysis elsewhere. Viral target extraction from paper is simple and economical, with low risk of contamination and containing less PCR inhibitors of plant and insect origin. In addition to this, the imprints or squashes can be safely used with quarantine plant viruses or insects. The success in amplifying CTV targets from pre-squashed aphids opens new possibilities for epidemiological studies and for better understanding of the virus-vector relationships.

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