CITRUS TRISTEZA VIRUS

Comparison of ELISA and PCR for the Sensitive Detection of Citrus Tristeza Virus (CTV) in Pooled Leaf Samples From Sweet Orange Groves with a Low Incidence of Infection

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ABSTRACT. A sensitive detection method is required if pooling of samples is chosen as a practical solution to the demands of large scale testing for virus in groves expected to have a low incidence of CTV. ELISA and PCR were compared for this purpose. Groves in the San Joaquin Valley of California in which all trees had been tested by ELISA were used as a source of infected and non-infected samples. ELISA negative tissues from individual trees were pooled and used to dilute ELISA positive tissue at dilutions ranging from 1/8 to 1/250. Distal leaf tips (250 mg, PCR) and proximal petiole tissue (170 mg, PCR and ELISA) were analyzed. ELISA was able to detect CTV in these tissue mixtures at a maximum dilution of 1/30. RT-PCR was able to detect CTV at maximum dilutions of 1/200 for leaf tip tissue and 1/250 for petiole tissue. Dilutions greater than 1/250 present problems of excessive tissue sample weight (approximately 50 g). These results suggest a potential for PCR detection in an eradication program, especially where the majority of groves are likely to be free of CTV.

The Central California Tristeza Eradication Agency (CCTEA) is attempting to find and remove all citrus trees infected with citrus tristeza virus (CTV) in the San Joaquin Valley area of California composed of Tulare, Kern, and Fresno counties. ELISA is the standard method utilized by the CCTEA for the detection of CTV. Groves are initially tested by ELISA, and typically 5% of the trees are sampled. Groves with CTV infection levels greater than 2% are resampled and every tree is tested. Trees testing positive by ELISA are confirmed by a second test before removal (1).

It is estimated that less than 0.4% of the total number of trees in the San Joaquin Valley are infected with CTV (approx. 75,000 infected trees out of 20 million total). In 1992-93, 14% of the groves tested at the initial 5% survey level were found to have infected trees, while 86% were CTV free. This is no doubt largely due to the planting of virus-free plants from nurseries certified by the Citrus Clonal Protection Program (5). Since such a large number of groves apparently are free of CTV it would be desirable to use a sensitive assay that would enable the accurate testing of samples pooled from a large number of trees for the initial survey. In this way negative groves would be identified with minimal effort and cost and a larger number of trees could be sampled. Those groves in which CTV was detected could then be tested on an individual tree basis by ELISA for final identification and removal of infected trees. The polymerase chain reaction (PCR) (8) is an assay that would enable such pooling. We present here the results of comparing ELISA and PCR assays for the detection of CTV in diluted samples from different tissue types.

MATERIALS AND METHODS

Experimental dilution series using liquid extracts. Sweet orange trees maintained in a green-

house at the University of California, Riverside, were used as the CTV positive and negative source plants for an experimental dilution series. The positive source was a plant known to harbor a high titer infection of a severe strain of CTV, and the healthy source was one tested repeatedly by ELISA to ensure that it was free from CTV. Standard double-antibody sandwich, indirect ELISA (2) was carried out using a polyclonal goat anti-CTV coating antibody (IgG, 1 μg/ml), antigen sample (see below for preparation and dilution), a polyclonal rabbit anti-CTV secondary antibody (1/3,000) (6), commercially obtained goat anti-rabbit/alkaline phosphatase conjugate (Sigma), and p-nitrophenyl phosphate (1 mg/ml, Sigma) as the substrate. All antibodies and protocols used were those used by the CCTEA to simulate their test method. The only exception was that antigens were prepared by grinding 0.5 g petioles from the positive and negative trees individually in 5 ml phosphate-buffered saline, pH 7.4 containing 0.5% Tween-20 and 2% (w/v) polyvinylpyrrolidone (PVP) using a Tek-Mar Tissuemizer (Cincinnati, OH 45222) (1/10, tissue/buffer) while CCTEA uses a Kleco Pulverizer (Garcia Machine, Visalia, CA 93277), and includes 0.2% bovine serum albumin. The infected sample liquid was diluted with the healthy extract such that the final infected tissue dilutions were 1/100, 1/1,000, 1/10,000, and 1/50,000.

After final development and recording of the absorbance values for the ELISA, the plates were washed with PBS (no Tween or PVP) and immunocapture PCR (IC-PCR) was performed using the RNA inside of virions trapped on the ELISA plate (7) as the template. One hundred μl of a reverse transcriptase-PCR (RT-PCR) mix composed of 0.4 mM dNTPs, 200 ng each of primer 64 (5'-TGACATTAGTAACTACGACATCATCAGCCC-3') and primer 65 (5'-ATGACGACGCCACGGGTATAACGTACACTC-3') (3,9), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 (1X Thermophilic Buffer, Promega), 2.5 mM $MgCl_2$, 10mM dithiothreitol, 10 U RNasin (Promega), 15 U AMV reverse transcriptase (Promega), and 2.5 U Taq DNA polymerase (Promega) were placed in each well of the ELISA plate to be assayed. The plate was incubated at 37°C for 1 hr, then the sample liquid was transferred to a microfuge tube, placed in a thermocycler (EriComp, San Diego, CA), incubated an additional 15 min at 42°C and then denatured for 2 min at 95°C. Forty amplification cycles consisting of 95 C/1'; 50 C/1'; 72 C/2' were performed followed by a final 5 min. at 72°C.

Dilution series using pooled field tissue samples. A representative sample collected by the CCTEA in the field is composed of eight leaves harvested from around the perimeter of a single tree. These leaves are stacked then wrapped with a rubber band to form a "bundle". Leaf bundles from commercial sweet orange trees which had tested positive at the CCTEA for CTV by ELISA were sectioned as follows: 1) the stem-end 5 to 7 mm of petioles; 2) the remainder of the petioles and 3 to 5 mm of leaf laminar tissue containing the midrib; and 3) the distal 8 to 10 mm of the leaf tips. Tissue types 1 and 3 were used for RT-PCR and type 2 was assayed by ELISA followed by IC-PCR as described above. Each tissue type from a single CTV-positive bundle was mixed with equivalent tissues from several CTV-negative bundles in ratios of 1/8, 1/15, 1/30, 1/60, 1/100, 1/150, 1/200, and 1/250. Total tissue weights ranged from 2-68 grams depending on the tissue type and pooling ratio. Each CTV positive bundle and a 20 g aliquot of pooled CTV negative tissue were also tested by RT-PCR to confirm their respective status.

Total nucleic acid (TNA) extracts were prepared by grinding each pool of tissue (1/8-1/250) in liquid nitrogen, adding 1 volume (1 g = 1 ml) of 100 mM Tris-HCl, pH 7.5, which contained 2 mM EDTA, and 2% SDS (TES) and 1 volume phenol:chloroform (2:1). The extract was heated at 70°C for 5 min. and the aqueous phase was recovered. Four 100 µl aliquots were removed, further purified by Sephadex G-50-80 column chromatography (4), and pooled. Twenty µl of the column-purified TNA was heat denatured at 65°C for 10 min then added to 80 µl of an RT-PCR mixture as described above. The samples were placed in the thermocycler and incubated at 42°C for 45 min., then denatured and amplified for 40 cycles as described above.

Detection of PCR products. 5 µl of each PCR product was added to 100 µl of denaturing solution (1.25 ml TE buffer, 2.5 ml 20X SSC, and 1.0 ml 37% formaldehyde) (3), heated at 55°C for 20 min., cooled on ice, and blotted to either a nitrocellulose membrane (MSI, Westborough, MA) for the radioactive probe or a nylon membrane (ZetaProbe, Bio-Rad, Richmond, CA 94804) for the non-radioactive probe, using a slot blot apparatus. The membrane was then baked for 30 min. at 80°C under vacuum. The probe oligonucleotide CTV-P3 (5'- CTGATTTAGAATGTGCTGTGTACA-3', genomic sense, which binds near the 3' end of the coat protein gene) (3) was end-labeled using either ^{32}P-ATP and T4 polynucleotide kinase (4) or the LightSmith II system (Promega). Membranes were prehybridized, hybridized, and washed as detailed by Lair et al. (3) for the radioactive probe or as outlined in the LightSmith II protocol manual for the chemiluminescent probe. The blots were exposed to Fuji RX x-ray film for 18-20 hrs/-80°C (radioactive probe) or 5-20 min./37°C (non-radioactive probe) and developed using a Konica QX-70 automatic processor.

RESULTS AND DISCUSSION

Liquid extract dilution series The initial study was set up to compare directly the detection limits of ELISA and IC-PCR when using infected and healthy samples which were individually extracted, then mixed as liquid extracts at specific ratios. ELISA was able to detect CTV in infected sap from a high titre source when it was diluted with healthy sap up to a 1/1,000 dilution (Figure 1, lane A). Absorbance values for the 1/10,000 and 1/50,000 dilutions were below the positive threshold limit for ELISA which was defined as 2.5 times the absorbance value of the healthy control following the conservative practice of the CCTEA. However, based on the ELISA absorbance values for the 1/1,000 and 1/10,000 dilutions, it is conceivable that ELISA would have been able to detect CTV at dilutions of 1/5,000 or 1/8,000 had they been tested. IC-PCR performed after the ELISA results were obtained detected CTV at dilutions up to 1/10,000 using the radioactive probe, and up to 1/50,000 when using the chemiluminescent probe (Fig. 1, lanes B and C).

Large scale pooling of field samples Diluting individually prepared samples is quite different than pooling tissue and then extracting it. In the former it is almost certain that target RNA will be present in the dilutions. In the latter however, since the infected tissue is mixed with the healthy tissue first, especially on a large scale, the odds of target RNA being isolated are reduced. Tissue pooling is the method that would be employed in an actual field experiment. When infected petioles were mixed with healthy petioles, ELISA was able to detect CTV at poolings up to 1/30 (Fig. 2, panel A).

CTV was not detected by IC-PCR from the pooled tissue samples, but was detected in unpooled infected

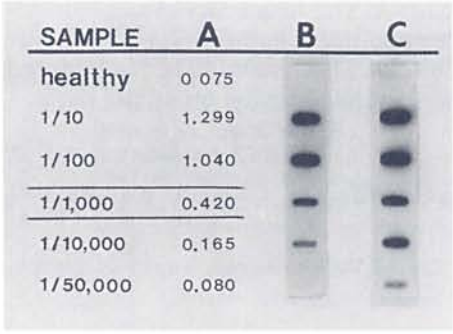

Fig. 1. ELISA absorbance values (A) and autoradiographs of blotted IC-PCR products hybridized with either a radioactive (B) or non-radioactive (C) CTV-specific probe. Final dilutions of CTV infected material or the healthy negative control are shown to the left of the figure. The ELISA cutoff value was 0.188 (2.5 times the healthy absorbance value). The highest dilution which had an absorbance value above this number is outlined.

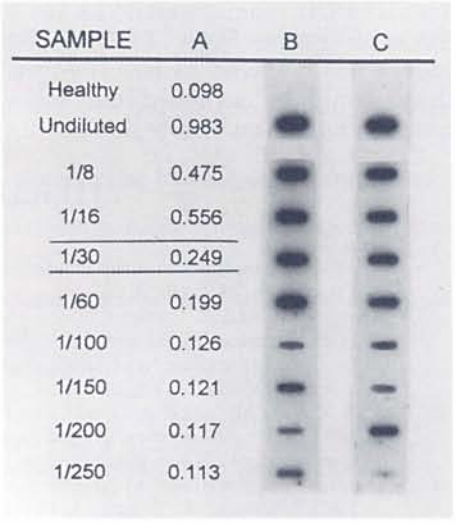

Fig. 2. Large scale pooling of tissue samples assayed by ELISA (A) and RT-PCR (B and C). Petioles were used for A and B and leaf tips were used for C. Tissue dilutions tested are shown to the left. The ELISA cut-off value was 0.245 (2.5 times the healthy absorbance value). The highest dilution which had an absorbance value above this number is outlined.

samples (data not shown) and in liquid extract dilutions with healthy sap when ELISA values for the same samples were indistinguishable from a healthy control sample (Fig. 1), so the method is sound. One possible explanation for the failure of IC-PCR on pooled samples is that the tissue was ground in liquid nitrogen, a method that was appropriate for the large amount of tissue created by the pooling. This method may provide a sample that is unsuitable for IC-PCR. Until the basis for the unexpected loss of sensitivity when using pooled tissue is determined, this technique would appear to be valuable for CTV samples which gave borderline ELISA results with samples prepared in the traditional way, but not for testing pooled samples from large numbers of trees.

RT-PCR was able to detect CTV in pooled petioles at 1/250, the highest dilution tested (Fig. 2, panel B). When leaf tips were used as the pooled tissue RT-PCR detected CTV at 1/200 (Fig. 2, panel C). Undiluted infected samples gave strong ELISA and RT-PCR reactions (Fig. 2, panels A-C), while a representative aliquot of the healthy tissue used for pooling tested negative by both ELISA and RT-PCR (Fig. 2, panels A-C).

Only a limited benefit would be achieved by using ELISA to test pooled samples since, at best, only 30 samples could be pooled before it becomes unreliable. However, RT-PCR would allow a significant reduction of sample handling since CTV could be detected when up to 250 leaf petiole samples were pooled. Leaf tips also gave good results when tested by RT-PCR and have the added benefit of being "leftover" tissue saved after the petioles are used for ELISA at the CCTEA. Conservatively, pooling could be restricted to 100-150 samples to allow for low titer infections. This number would also make sense for use at the CCTEA since the initial 5% survey from a typical 2,000 tree grove yields 100 samples for testing. Instead of 100 ELISA samples being assayed, a single TNA extraction

and RT-PCR reaction could be used for each grove. Since 86% of the groves are believed to be CTV-free, these could be screened out more quickly than is currently done using ELISA. The feasibility of implementing pooling of samples for RT-PCR at the CCTEA should be considered and perhaps tested on a trial basis.

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