Detection of Tomato Ringspot Virus by Polymerase Chain Reaction

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ABSTRACT

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A polymerase chain reaction (PCR) protocol has been developed for the detection of tomato ringspot virus (TomRSV) in herbaceous and woody plant tissues. This PCR test detected all major TomRSV serogroups (A through E) and can detect as little as 60 pg of target sequence in *Malus sylvestris* leaf tissue. The oligonucleotides selected for the TomRSV PCR primed a 449-nucleotide region of the putative viral polymerase gene and did not react to any of the healthy plant tissues tested. The high sensitivity and specificity of TomRSV PCR give researchers a new and powerful technique to study this important plant virus.

Additional keywords: ELISA, GVYV, PYBMV

Tomato ringspot virus (TomRSV), a member of the nepovirus group, is found in many perennial crops in North America and to a lesser extent in Europe, Asia, Australia, and South America (20). TomRSV is vectored by several species of *Xiphinema* Cobb (3,4,7–9,12) and is also transmitted via vegetative propagation, seed, and, rarely, pollen (20).

TomRSV has been shown to cause mild to severe economic loss in many perennial fruit crops including *Malus* Mill. (22,24) and *Prunus* L. (19), and is an agent of concern for many countries that produce or import nursery stock. Phytosanitary certification often requires that such nursery stock be tested and found free from TomRSV and other viruses by regulatory agencies. State agencies such as the Oregon Department of Agriculture regularly conduct large-scale sampling of commercial production operations to screen for TomRSV. Current detection techniques for Oregon's certification program include serological tests, graft-indexing, and sap transmission to indicator plants. Recent advances in enzymatic polymerization have demonstrated successful detection of nepovirus nucleic acids in woody plants (18). The potential of polymerase chain reaction (PCR) for the rapid detection of low copy amounts of TomRSV nucleic acid would be helpful for both epidemiological studies and for a rapid check of plant material in certification programs.

I report here on the polymerase amplification of a section of putative TomRSV polymerase gene from purified viral preparations as well as from infected herbaceous and woody plants. The sensitivity of the procedure was evaluated and compared with double antibody sandwich (DAS)–indirect enzyme-linked immunosorbent assay (ELISA) in the presence of *M. sylvestris* tissue extracts.

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MATERIAL AND METHODS

TomRSV sources. The TomRSV isolates were chosen to represent the five reported serotypes of TomRSV (A, B, C, D, and E) (1) and also represent a variety of isolates of economic significance in perennial crops. The isolates included the following: grapevine yellow vein (GVYV) prune brownline (PBL), and peach yellow bud mosaic virus (PYBMV) from California (A. Rowhani); Chickadee, Apricot, and Winchester isolates from New York (D. Gonsalves); and two raspberry isolates, one from British Columbia and SL-4 from Oregon (G. Milbrath).

Total nucleic acid extraction. Tissue samples for PCR were prepared either by phenol-chloroform (PC) extraction in 0.1 M Tris buffer, pH 8.5 (11) or by differential centrifugation (DC) in 0.1 M phosphate buffer, pH 7.6 (6). In addition to these purification procedures, some samples were further purified with either a treatment of cetyltrimethylammonium bromide (CTAB) (13) or activated charcoal (18). Two microliters of the resulting solution was then used in the reverse transcription (RT) reaction.

Primers used in PCR amplification. Primers were designed from TomRSV viral sequence information (17). Primers included U1, (5′ to 3′) GACGAAGTTATCAATGGCAGC (nucleotides [nt] 1,078 to 1,098) and D1, TCCGTCCAATCACGCGAATA, (nt 1,506 to 1,527) of the putative viral polymerase gene. This putative enzyme sequence was selected as the amplification target assuming the likelihood of highly conserved sequences between isolates, as compared to the antigenically distinct and possibly more variable coat protein coding sequences.

RNA transcription. All samples in this study were reverse transcribed independently of the PCR reaction. A 2-µl aliquot of sample was added to an RT master mix containing 10 µg/ml of D1 primer. The RT master mix contained 100 units of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, Md.) per sample and dNTPs, salts, and buffer as per the manufacturer's directions. The total reaction volume of 20 µl received a 10-µl overlay of mineral oil and a brief centrifuge spin at 4,000 rpm. The reaction was performed in a Perkin-Elmer Corp. (Norwalk, Conn.) model 4600 thermocycler at 37 to 40°C for 50 min., followed by 95°C for 5 min.

PCR amplification of TomRSV. The PCR reaction used 1 to 2 µl of RT product, 1 µM each of primers U1 and D1, and an optimized concentration of magnesium chloride (1.5 mM). Several polymerases were used; however, 1 unit of *Taq* polymerase (Promega Corp., Madison Wis.) per reaction generally gave stronger and more consistent results with a minimum of streaking. The total reaction volume of 75 µl contained dNTPs, buffer, and detergent per manufacturer's directions. Samples were pre-heated to 77 to 80°C for 3 to 4 min before *Taq* was added to minimize nonspecific priming. After the reaction mixture was complete, the temperature was raised to 94°C for 4 min to denature the template. The PCR reaction began with a 94°C denature for 1 min, followed by a 55 to 60°C anneal for 2 min and a 72°C extension for 2 min. Normally, 35 to 40 cycles were performed, followed by a 5 to 10 min extension at 72°C and then lowered to a holding temperature of 1°C.

Gel analysis of RT-PCR products. Fifteen microliters of each sample was mixed with 10× Ficoll-EDTA-Tris loading buffer and electrophoresed in a 2.0% Metaphore agarose gel (FMC BioProducts, Rockland, Maine) in 0.5× Tris-borate-EDTA buffer. The power supply was set to constant current at 40 mA and run for 1 to 1.75 h in pre-chilled buffer. The gels were stained in a 0.5 µg/ml ethidium bromide solution for 20 to 30 min, destained in water twice. Photographs were taken over a UV transilluminator with a 35-mm single-lens reflex camera set at F8 for 1 s. Restriction digests were obtained with 10 units/µl *Hin*fI enzyme in the presence of 1× buffer B (Promega) at 37°C for 1 h.

Sensitivity of PCR in distilled water and in apple sap. To determine relative sensitivity of the RT-PCR procedure, purified GVYV was diluted in distilled water to contain 1 µg/ml of RNA 1, assuming 44% RNA 1 content (20). The RNA was

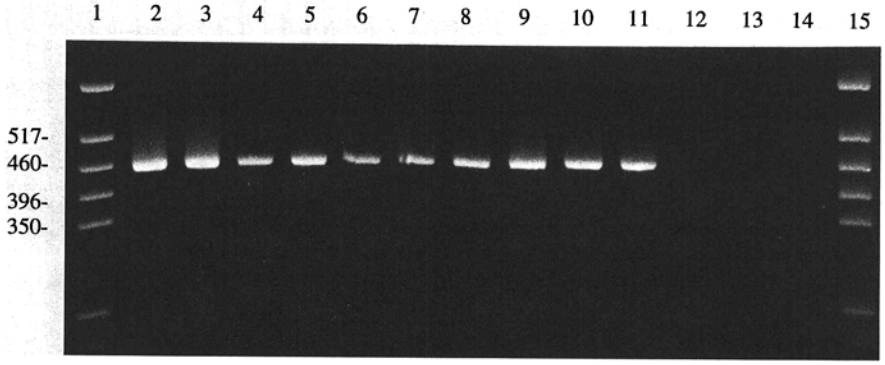

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products of amplified virus sources and controls. Lane 1, DNA ladder; lane 2, purified grapevine yellow vein virus (GVYV); lane 3, purified peach yellow bud mosaic virus (PYBMV); lane 4, Chickadee; lane 5, Winchester; lane 6, raspberry isolate (Oregon); lane 7, raspberry isolate (British Columbia); lane 8, GVYV in *Vitis labrusca*; lane 9, prune brownline (PBL); lane 10, Apricot; lane 11, infected dormant *Malus* bud tissue; lane 12, cherry rasp leaf virus (CRLV); lane 13, water blank; lane 14, healthy tissue (*Malus*); lane 15, DNA ladder.

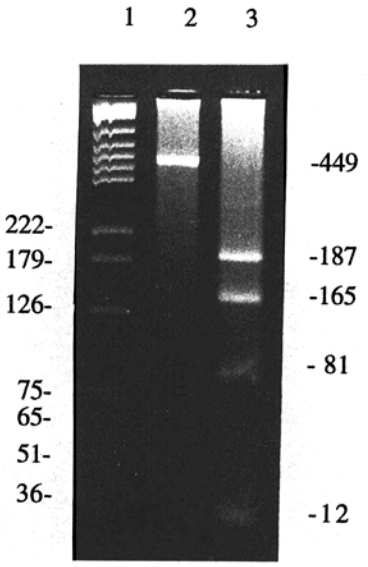

Fig. 2. Agarose gel electrophoresis displaying *Hin*fI digest of PCR product. Lane l, DNA ladder; lane 2, grapevine yellow vein virus (GVYV); lane 3, GVYV amplification product after restriction.

then added to 0.2 g of healthy apple tissue with 800 µl of extraction buffer and then processed by PC extraction. The same amount of purified GVYV was added to 800 µl of extraction buffer without any tissue and then processed by PC extraction as a sap-free control. Ten-fold serial dilutions were then aliquoted and polymerized using the primer pair U1-D1. These dilutions provided a range of concentrations of RNA 1 from 6.0×10^{-8} to 6.0×10^{-13} g per sample.

Comparison of RT-PCR and ELISA. Aliquots of macerated tissue from the two dilution series (with and without apple sap) containing purified GVYV were analyzed by DAS-ELISA. ELISA plates (Immulon 2, Dynatech Co., Chantilly, Va.) were coated with a commercially available TomRSV IgG at a 1:1,000 dilution in carbonate buffer per manufacturer's directions. Two wells were filled with 200 µl of sample containing 750 ng of GVYV coat protein, assuming 15% capsid protein content (20). Three additional pairs of wells were loaded with ten-fold dilutions of the first sample, providing a range of dilutions from 7.5×10^{-7} to 7.5×10^{-11} g per well of viral coat protein. Six wells were loaded with healthy apple tissue to establish a threshold absorbance value. Samples were considered positive if the absorbance values were greater than twice the mean of the healthy tissue (23). Alkaline phosphatase conjugate was added at a dilution of 1:1,000 per manufacturer's directions. Diethanolamine substrate buffer with 1 µg/ml p-nitrophenylphosphate was added to the plates per manufacturer's recommendations. The plates were then analyzed after 45 to 60 min by dual-wavelength mode on a Vmax plate reader (Molecular Devices, Menlo Park, Calif.).

RESULTS

Amplification products of TomRSV RT-PCR. Gel analysis of the RT-PCR of purified GVYV and PYBMV produced a single DNA product of the predicted length of 449 nt (Fig. 1). Additionally, TomRSV RT-PCR produced the predicted products for known positive plant samples infected with Chickadee, Winchester, and raspberry isolates, one in raspberry bud tissue and one in cucumber. GVYV was also detected in grape leaf tissue (*Vitis labrusca* L.), dormant *Malus* bud tissue, and *Chenopodium quinoa* Willd. Representative isolates from all major serogroups, A (Chickadee), B (GVYV), C (PYBMV and PBL), D (Winchester), and E (Apricot), produced the 449-nt product (Fig. 1). No PCR products were observed with the healthy tissue controls or in water blanks. Samples containing cherry rasp leaf virus (CRLV), a closely related nepovirus (14,21) also failed to produce any PCR products.

Restriction enzyme analysis using *Hin*fI yielded fragments of 187, 165, 83, and 12 nt (Fig. 2). These fragments are the size predicted from the sequence data (17) and confirm that the 449-nt amplification product is analogous to nt 1,078 to 1,527 of the putative viral encoded polymerase gene.

Effect of purification methods. TomRSV was easily detected in herbaceous hosts such as *C. quinoa* when either the PC or the DC procedure was used (data not shown). However, in perennial hosts such as grape or in apple tissue amended with purified TomRSV, the use of PC purification generally required further treatment with Tween 20 and activated charcoal with a period of freezing to produce a strong amplification signal. The CTAB treatment did not increase virus detection in these samples. The DC purification alone sometimes allowed for a strong PCR signal, but the results were inconsistent. Application of the Tween 20 or the CTAB to DC did not increase test sensitivity (data not shown).

Sensitivity of TomRSV PCR and comparison to DAS-ELISA. Serial dilution of samples from TomRSV-spiked apple leaf tissue showed that PCR is able to produce visually detectable signal to 6.0×10^{-11} g of template using PC and Tween treatment (Table 1). In contrast, DAS-ELISA was able to detect TomRSV-spiked apple leaf tissue with 7.5×10^{-7} g of coat protein, making the PCR at least 10,000 times more sensitive in these split sample comparisons (Table 1). The RT-PCR of purified TomRSV produced visually detectable signal with 6.0×10^{-13} g of purified tissue-free template, showing a 100-fold decrease in sensitivity in the presence of apple sap (Table 1). This decrease may be due either to the PC extraction procedure or an interference in the RT-PCR process by sap components as others have proposed (18). DAS-ELISA was able to detect 7.5×10^{-9} g of coat protein in the purified sample dilution series, which agrees closely with test sensitivity previously reported (6.1×10^{-9} g) (5). The ELISA detection endpoint for samples with apple sap was 100-fold lower than the detection endpoint for samples free from apple sap (Table 1).

DISCUSSION

The purpose of this work was to develop an RT-PCR protocol to detect multiple strains of TomRSV in both herbaceous and woody plants. While this protocol can be used for a number of applications, this procedure may be of particular value in host-pathogen systems in which standard serological testing is only partially effective. For example, ELISA is currently the preferred testing method in the certification of apple nursery stock. However, researchers have reported spurious reactions to nonviral antigens resulting in ELISA A_{405} values as high as 13 times that of the healthy control (15). Presumably, the poly-

Table 1. Comparison of detection sensitivities between DAS-ELISA[a] and RT-PCR[b] in serial dilutions of purified tomato ringspot virus in both apple sap and water.

		DAS-ELISA				RT-PCR	
Sample	Sample dilution	Capsid (ng/sample)	A_{405} mean	A_{405} SD	Results (+/-)	RNA 1 (pg/sample)	Results (+/-)
GVYV + water	10^{-8}	75.0	0.393	0.030	+	60	+
GVYV + water	10^{-9}	7.5	0.213	0.049	+	6	+
GYVY + water	10^{-10}	0.75	0.125	0.018	–	0.6	+
GVYV + water	10^{-11}	0.075	0.102	0.010	–	0.06	+
Healthy apple	. . .	0	0.072	0.013	–	0.006	–
GVYV + sap	10^{-7}	750.0	0.279	0.055	+	600	+
GVYV + sap	10^{-8}	75.0	0.016	0.018	–	60	+
GVYV + sap	10^{-9}	7.5	0.018	0.013	–	6	–
GVYV + sap	10^{-10}	0.75	0.024	0.033	–	0.6	–
Healthy apple	. . .	0	0.026	0.009	–	0.06	–

[a] Double antibody sandwich–enzyme-linked immunosorbent assays (DAS-ELISA) were conducted on apple sap and water containing serial dilutions of grapevine yellow vein virus (GVYV). Samples were considered positive (+) if the absorbance values (A_{405}) were greater than twice the mean of the healthy tissue.

[b] Reverse transcription and polymerase chain reaction (RT-PCR) procedures were conducted on aliquots of the serial dilution series used in DAS-ELISA. The final amount of sample used in the PCR test was 0.15% of the volume used in corresponding DAS-ELISA sample. Samples were considered positive (+) if a visible product of 449 nucleotides was resolved on an agarose gel.

clonal antisera used in the ELISA reacted to a plant-produced antigen that was prevalent in leaf tissue. As demonstrated here, the U1-D1 primed RT-PCR has yet to produce a PCR product in the presence of healthy apple controls. In this case, PCR could be used to re-test the seropositive samples and either confirm or refute the ELISA results. The PCR could also provide the recommended independent check on the ELISA A_{405} values and assist in setting a non-arbitrary absorbance threshold (23).

Another issue with assaying TomRSV by ELISA is test specificity. Antigenic variation in TomRSV serotypes requires the use of at least two different polyclonal or monoclonal antisera to detect all reported isolates of the virus (1,16). In order to provide a thorough screening, two TomRSV ELISA tests must be conducted for each sample, effectively doubling the costs of a survey or certification for this virus. To date, all isolates of TomRSV tested with RT-PCR primed by U1-D1 have produced a 449-nt product. Since the primer pair anneals to sequence analogous to the putative viral polymerase gene, the reaction is not dependent on antigenically variable capsid protein. This could allow for a single test per sample and save a great deal of effort and cost in TomRSV surveys.

An additional problem with ELISA in apples is that the detectable distribution of TomRSV capsid protein within a given apple tree varies with tissue type, season, and tree age (2). It is possible to collect leaves from an infected plant and not generate an A_{405} value high enough to be considered positive (2). It is unclear whether a low A_{405} value represents a true absence of TomRSV, a lack of sensitivity of the ELISA due to very low titer, or a detrimental effect of the apple sap on the ELISA as reported here. While this observation is limited to the interactions of a single polyclonal antiserum, apple cultivar, and virus isolate, this apparent interference effect of apple sap could be a cause of concern for the detection of low titer infections by ELISA and should receive further investigation.

The sensitivity of this RT-PCR may allow for increased detection of TomRSV compared with samples tested by ELISA, and may also allow for a high degree of aggregate sampling where practical, such as in rootstock beds or bundles of finished trees. Additionally, these primers could be added to primers for other viruses and other agents of concern, providing a multiplex PCR test.

While this RT-PCR test by itself is not as sensitive as some bioassays or cDNA probes (10), the speed and ease of the procedure make it a valuable new tool to study many aspects of TomRSV epidemiology. Future work will focus on the ability of this RT-PCR test to detect low titer infections in leaf tips, pollen, and seed as well as detection in nematode vectors.

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