Detection of Potato Leafroll and Strawberry Mild Yellow-Edge Luteoviruses by Reverse Transcription-Polymerase Chain Reaction Amplification

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ABSTRACT

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DNA primers were constructed based on the nucleotide sequence of the coat protein gene of potato leafroll luteovirus (PLRV) and utilized for cDNA synthesis and polymerase chain reaction (PCR) amplification of a 487-bp DNA fragment from nucleic acid extracts of PLRV-infected tissue, and an approximately 500-bp DNA fragment from the luteovirus associated with strawberry mild yellow-edge-infected tissue. The amplified DNA fragments were identified by hybridization analysis with a cDNA probe for the coat protein gene of PLRV and differentiated by restriction fragment length polymorphism analysis. Reverse transcription (RT)-PCR assays were developed for the detection of PLRV in nucleic acid extracts of infected potato leaves and tubers, and in viruliferous aphids. The luteovirus-specific DNA was absent from amplified extracts of uninfected potato or nonviruliferous aphids. The RT-PCR assay for PLRV is more sensitive than existing detection methods and detects PLRV in plant hosts or insect vectors without requiring large samples or molecular hybridization.

Potato leafroll luteovirus (PLRV) causes a serious disease of potato (*Solanum tuberosum* L.) that results in significant crop losses worldwide (32,36). It is transmitted by the green peach aphid (*Myzus persicae* (Sulzer)) in a persistent, circulative manner and is limited to the phloem tissue of the host plant (2,11). PLRV has an isometric morphology and a messenger-sense RNA genome that has a small genome-linked protein and no stretches of polyadenylate (24). The nucleotide sequence and genomic organization of PLRV isolates from Europe, North America, and Australia have been determined (16,25,42). At least 93% nucleotide-sequence homology has been observed among PLRV isolates (16,23).

Strawberry mild yellow edge (SMYE) is an aphid-borne disease that causes severe loss of plant vigor, yield, and fruit quality when its causal agent(s) occurs in a complex with aphid-borne strawberry viruses such as mottle, veinbanding, and crinkle (3). It is distributed worldwide in cultivated strawberries and is among the 50 most frequently cited plant diseases in the quarantine regulations of 124 countries (14). Strawberry mild yellow-edge virus may be a complex of the viruses SMYE-associated potexvirus (SMYEAV) (12) and a putative luteovirus (SMYEV). SMYE is believed to be caused by SMYEV based on symptomatology, lack of mechanical transmission, persistent transmission of the virus in a circulative manner by the strawberry aphid *Chaetosiphon fragaefolii* (Cockerell) (27), and localization of 22–25 nm isometric virus particles in phloem cells of infected strawberry plants (47). Also, SMYEV has a distant serological relationship with beet western yellows luteovirus (38).

The biology of the luteoviruses associated with PLRV and SMYEV complicates their identification in infected tissue because these viruses are confined to phloem tissue, occur in extremely low concentrations, and are not transmitted mechanically (34). Consequently, conventional detection methods may not be sensitive enough for diagnostic, epidemiological, or genetic studies, or studies of virus–host–insect interactions. Our recent investigations of several fruit-crop viroids, plum pox potyvirus and grapevine closterovirus A, (7,9,10,19,20,28,45, 46), as well as investigations of others on the potato spindle tuber viroid (15), potyviruses (17,18,43), nepoviruses (1), and luteoviruses (13,34), have shown that the reverse transcription-polymerase chain reaction (RT-PCR) detection method is more sensitive than other methods. Based on these findings, we have tested RT-PCR to amplify a PLRV coat protein sequence in potato and insect extracts, and an SMYEV coat protein sequence in strawberry extracts. This paper describes the detection and identification of PLRV, from the total nucleic acids of infected potato plants and viruliferous green peach aphids, and of SMYEV from the nucleic acids of infected strawberry extracts. Preliminary reports of this work have been presented (4,29; A. Hadidi, M. S. Montasser, L. Levy, and R. H. Converse, *unpublished*).

MATERIALS AND METHODS

Potato and PLRV sources. Several PLRV-infected and uninfected potato cultivars were used in this study, including Penobscot, Katahdin, Green Mountain, and BelRus. Unless otherwise stated, plants of these cultivars were maintained in a greenhouse with an ambient temperature range of 15–30 C. High-pressure sodium lights (8,000–10,200 lx) were used as supplemental lighting to maintain a 16-hr day length. The PLRV vector, *M. persicae,* was maintained on PLRV-infected potato or *Datura stramonium* L. plants for 10–14 days before aphid processing. These insects were determined to be viruliferous by their transmission of PLRV to uninfected plants. Nonviruliferous aphids were maintained on tobacco (*Nicotiana tabacum* L.) plants. A characterized Beltsville isolate of PLRV, maintained by R. W. Goth, was used in most of this investigation. Isolates from Poland and Egypt were also used to confirm results obtained with the Beltsville isolate.

Source of SMYE-diseased strawberry tissue. Leaf samples were collected from cultivated strawberry *Fragaria* × *ananassa* Duchesne 'Totem' that were naturally infected with strawberry mild yellow-edge virus. Plants were cultivated for 4 yr in a large commercial field in Boring, in the Willamette Valley in Oregon.

Nucleic acid extraction from plant and insect tissue. Total nucleic acids from healthy and infected plant tissues and from viruliferous and nonviruliferous

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aphids were isolated as previously described (6,44,45). However, total nucleic acids from potato or strawberry leaf tissue was obtained by a different procedure than total nucleic acids from potato tuber or aphid tissue. Briefly, tissue from potato leaf, potato tuber, or strawberry leaf (approximately 0.25 g) or insects (50–100 aphids) was frozen with liquid nitrogen and ground to a fine powder. Ground leaf tissue was extracted in 3 ml of extraction buffer (0.1 M glycine-NaOH, pH 9.0; 50 mM NaCl; 10 mM EDTA; 2% sodium dodecyl sulfate [SDS]; and 1% sodium lauryl sarcosine) (44), 3 ml of water-saturated phenol, and 2 ml of chloroform/isoamyl alcohol (24:1). The aqueous phase of each sample was recovered by low-speed centrifugation; and nucleic acids were precipitated by the addition of 0.10 volume of 7.5 M ammonium acetate, pH 5.5, and 2.5 volumes of ethanol, and kept at −70 C for at least 2 hr or at −20 C overnight. Nucleic acid pellets were washed twice with 70% ethanol, dried in vacuo, and dissolved in water or TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The nucleic acid concentration was determined spectrophotometrically (22). Total nucleic acids from strawberry tissue were further purified on RNase-free ELUTIP-r minicolumns containing nucleic acid binding matrix (Schleicher and Schuell, Keene, NH) as suggested by the manufacturer, with a modification in the low-salt buffer. The minicolumns were hydrated and equilibrated at room temperature for 2 hr with 5 ml of "low salt" buffer containing 20 mM NaCl; 20 mM Tris-HCl, pH 7.4; and 1.0 mM Na_2EDTA. Total nucleic acids (100 μg) was dissolved in 2–3 ml of the low-salt buffer and then passed slowly through the minicolumn. The minicolumn was eluted once with 5 ml of the "high salt" buffer (1.0 M NaCl; 20 mM Tris-HCl, pH 7.4; and 1.0 mM EDTA). Nucleic acids were precipitated by the addition of absolute ethanol at −70 C for at least 30 min followed by centrifugation at 12,000 *g* for 15 min at 4 C. Each pellet was washed once with cold 70% ethanol to remove any traces of salt, dried in vacuo, dissolved in deionized water, and the nucleic acid concentration determined.

An alternative method was used for extraction of nucleic acids from potato tubers and aphids. Each sample of ground potato tuber tissue or aphids was extracted for 20 min in 5 ml of the glycine extraction buffer, as described above. This preparation was extracted with 5 ml of Tris-HCl-buffered phenol, pH 7.5–8.0, containing 0.1% 8-hydroxyquinoline and 0.2% (v/v) 2-mercaptoethanol, for 15 min and then mixed for an additional 15 min with 5 ml of chloroform (6,45). Samples were centrifuged at 3,008 *g* for 15 min, and the aqueous phase of each sample was recovered. Total nucleic acids were precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 3 volumes of absolute ethanol, incubated at −70 C for a minimum of 2 hr, and centrifuged at 12,000 *g* for 30 min at 4 C. The resulting pellets were dissolved in 1 ml of TE buffer and reprecipitated with ethanol and sodium acetate as previously described. The pellets were dissolved in 500 μl of TE buffer, and the nucleic acid concentration was determined spectrophotometrically.

Viral cDNA synthesis and amplification. Total nucleic acids of potato or strawberry tissue (1–3 μg) or aphids (1 μg) were added to 140–420 μM of a 22-mer primer ([5′-GCACTGATCCTCA GAAGAATCG-3′] complementary to PLRV RNA nucleotides 4,103–4,124 in the coat protein region [42]), 6 μl of 5× first-strand cDNA buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$; and 50 mM dithiothreitol [DTT]), and water to a final volume of 30 μl. The mixtures containing nucleic acids from plant tissue or aphids were heated at 65 C and 100 C, respectively, for 5 min; chilled on ice for 2 min; and incubated at room temperature for 1 hr to allow primer annealing to the RNA template. A total of 18 μl of cDNA reaction mixture (4 μl of 5× first-strand cDNA buffer, 5 μl of 0.3 M 2-mercaptoethanol, 2.5 μl of 10 mM each deoxynucleoside triphosphate [dNTP], 1 μl of RNasin [40 U/μl, Promega Corp., Madison, WI], and 5.5 μl of sterile deionized water) and 2 μl of cloned Moloney murine leukemia virus reverse transcriptase (200 U/μl, GIBCO BRL Life Technologies, Gaithersburg, MD) were mixed with the annealing reaction mixture and incubated at 42 C for 2.5 hr.

Aliquots (5 μl) of the reverse transcription reaction were transferred to tubes each containing 45 μl of polymerase chain reaction buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; and 0.01% [w/v] gelatin), 200 μM each dNTP, 1.2 μM of the above 22-mer primer, 1.2 μM of a 22-mer primer (5′-AAGAAGGCGAAGAAGGCAATCC-3′ homologous to PLRV RNA nucleotides 3,638–3,659 in the coat protein region [42]), and 0.5 μl of AmpliTaq DNA polymerase (5 U/μl, Perkin-Elmer Cetus Corp., Norwalk, CT). Samples were vortexed briefly and overlaid with 50 μl of mineral oil to prevent evaporation. Polymerase chain reactions were performed for 35–40 cycles in a programmable DNA thermocycler (Perkin-Elmer Cetus Corp.). The reactions involved a program of denaturation at 94 C for 1 min, annealing at 55 C for 2 min, and extension at 72 C for 3 min, with a final primer extension cycle of 7 min.

Analysis of RT-PCR amplified products. Aliquots (5 μl) of the RT-PCR amplified cDNA fragments were either directly analyzed by electrophoresis or digested before analysis with 2 units of *Sau*3AI or *Sin*I in a total volume of 20 μl at 37 C for 2 hr as recommended by the manufacturer (GIBCO/BRL Life Technologies). Electrophoresis was through 6% polyacrylamide vertical slab gels (11 × 14 × 0.15 cm) at 120 V for 2.5 hr in 1 X TBE buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM Na_2EDTA, pH 8.3). Separated RT-PCR DNA fragments were visualized with either silver nitrate (10) or ethidium bromide staining. pGEM DNA molecular weight markers (Promega, Madison, WI) consisting of 15 DNA fragments ranging in size from 2,645 to 36 bp were used to determine the size of RT-PCR amplified DNA products.

Cloning of a fragment of cDNA from the PLRV coat protein gene. PLRV isolate L-7 from Poland (39) was purified from infected potato tissue by density gradient centrifugation essentially as described (40). PLRV RNA was isolated from purified virions by treatment with SDS and proteinase K (26) and was polyadenylated at the 3′ end by poly(A) polymerase (30). cDNA molecules were synthesized according to Gübler and Hoffman (5) and then blunt end-ligated in the dephosphorylated *Sma* I-digested plasmid vector pEMBL18+ as described (22). Recombinants were used to transform competent cells of *Escherichia coli* DH5αF′, and clones of transformed cells were identified by colony hybridization with 5′-labeled PLRV RNA. The cDNA of one of these clones, pLM26, was sequenced (L. J. Skrzeckowski, *unpublished*) and found to be 422 bp in length. Based on sequence and genetic organization of PLRV RNA reported by van der Wilk et al (42), recombinant plasmid pLM26 represents about 68% of the coat protein gene and covers the central part of the corresponding open reading frame 4, ending at nucleotide 4,147. This recombinant plasmid was labeled with [α-^{32}P]dCTP (3,000 Ci/mmole) by nick translation (Nick translation kit, GIBCO BRL) (33) and used as a molecular probe.

Southern blot hybridization. Polyacrylamide gels containing separated RT-PCR cDNA fragments were soaked twice in 0.4 M NaOH/0.6 M NaCl for 15 min each to denature the DNA, equilibrated twice in TAE buffer (40 mM Tris-HCl, pH 8.0; 20 mM sodium acetate; and 2 mM Na_2EDTA) for 5 min each, and electrotransferred to TAE-equilibrated Nytran nylon membranes (Schleicher and Schull, Keene, NH) at 0.6 mA for 16 hr at 4 C. Transferred nucleic acids were cross-linked to membranes by irradiation in a UV cross-linker (Stratagene, La Jolla, CA). Membranes were prehybridized, hybridized with the ^{32}P-labeled nick-translated PLRV cDNA in a Hybaid minihybridization oven (National Labnet Co., Woodbridge, NJ), washed, and exposed to X-ray film with intensifying screens at −70 C, as previously described (8) except that

RNase was eliminated during the washing steps.

RESULTS

Detection of PLRV and SMYEV in infected plant tissue by RT-PCR. Fig. 1 shows a polyacrylamide gel electrophoretic analysis of amplified cDNA fragments of PLRV and SMYEV. The size of the PLRV-amplified cDNA fragment was, as expected, 487 bp whether the starting nucleic acid was from purified PLRV RNA (lanes 2 and 8) or total nucleic acids of PLRV-infected potato leaf tissue (lane 3). The size of an SMYEV cDNA fragment, however, was slightly larger than 487 bp (lanes 5 and 9) because its electrophoretic mobility was slightly but distinctly slower than that of the PLRV cDNA. SMYEV cDNA was amplified from only ELUTIP-r minicolumn-purified total nucleic acids of infected strawberry tissue (lanes 5 and 9), but not from uninfected tissue (lane 4) or from total nucleic acids of infected and uninfected tissue before minicolumn purification (lanes 6 and 7). Thus further purification of total nucleic acids from SMYEV-infected tissue is required for successful RT-PCR amplification of SMYEV cDNA.

Fig. 2 shows Southern blot hybridization analysis, with a ^{32}P-labeled PLRV coat protein gene cDNA probe, of the PLRV cDNA of total nucleic acids from PLRV-infected potato leaf tissue (lane 1) and from purified PLRV RNA (lane 6), and the SMYEV cDNA of minicolumn-purified total nucleic acids from SMYEV-infected strawberry leaf tissue (lane 3). A major cDNA, of the expected size, and higher molecular weight cDNA products hybridized with the probe. However, the intensity of the hybridization signals of SMYEV cDNA (lane 3) was less than that of PLRV cDNA (lanes 1 and 6). Hybridization was not observed between the probe and minicolumn-purified total nucleic acids from uninfected potato leaf tissue (lane 2) or uninfected strawberry leaf tissue (lane 4), or between the probe and non-ELUTIP-r-purified total nucleic acids from SMYEV-infected tissue (lane 5).

The amplified cDNAs of PLRV and SMYEV were digested with the restriction endonucleases *Sau*3AI or *Sin*I and analyzed by electrophoresis on a 6% polyacrylamide gel. Fig. 3 shows restriction fragment length polymorphism (RFLP) analysis of the amplified cDNAs. The restriction profile of the fragments showed the presence of identical *Sau*3AI recognition sequences in both PLRV cDNA and SMYEV cDNA (lanes 4 and 8, respectively). However, the restriction profiles of *Sin*I-digested fragments of PLRV (lanes 2 and 5) and SMYEV (lanes 6 and 9) were different, indicating that the SMYEV fragment does not contain the same *Sin*I recognition sequences as the PLRV fragment.

Fig. 4 shows the distribution of PLRV RNA in PLRV-infected potato tubers, as determined by RT-PCR amplification of total nucleic acid extracts of different potato tuber tissues. The amplified 487-bp DNA fragment was detected in tuber tissues enriched in periderm and cortex (lanes 3 and 7), "eye" bud (lanes 4 and 8), and cortex and vascular ring (lane 5), but not in the perimedullary zone (lane 9) or in the medulla (pith) (lanes 6 and 10). Perimedullary tissue is the denser storage parenchyma that surrounds the pith.

RNA isolated from purified virions of PLRV has been reported to be associated with DNA (26,37). Gel electrophoretic analysis of RT-PCR amplified products of virion-purified PLRV RNA and total nucleic acids of PLRV-infected tissue, in the presence or absence of reverse transcriptase, showed that the 487-bp RT-

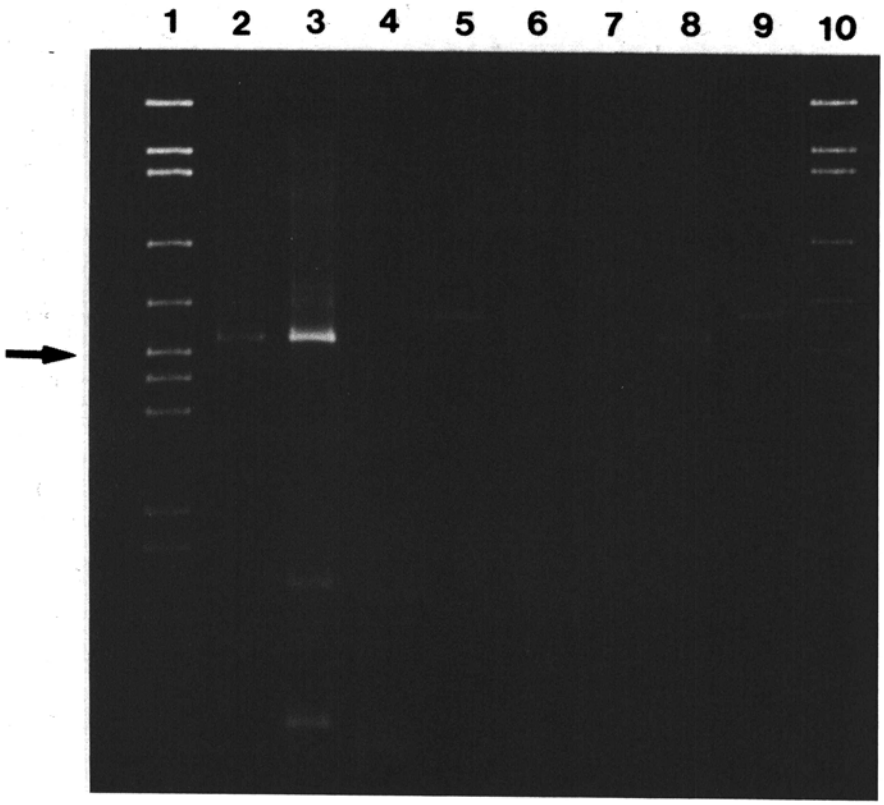

Fig. 1. Polyacrylamide gel electrophoretic analysis of reverse transcription-polymerase chain reaction (RT-PCR) amplified cDNA products of potato leafroll luteovirus (PLRV) and strawberry mild yellow-edge luteovirus (SMYEV). pGEM DNA markers, arrow indicates 460 bp (lanes 1 and 10); purified PLRV RNA (lanes 2 and 8); total nucleic acids of PLRV-infected potato leaf tissue (lane 3); minicolumn-purified total nucleic acids of uninfected strawberry leaf tissue (lane 4); minicolumn-purified total nucleic acids of SMYEV-infected strawberry leaf tissue (lanes 5 and 9); and total nucleic acids of uninfected and SMYEV-infected leaf tissue before minicolumn purification (lanes 6 and 7, respectively).

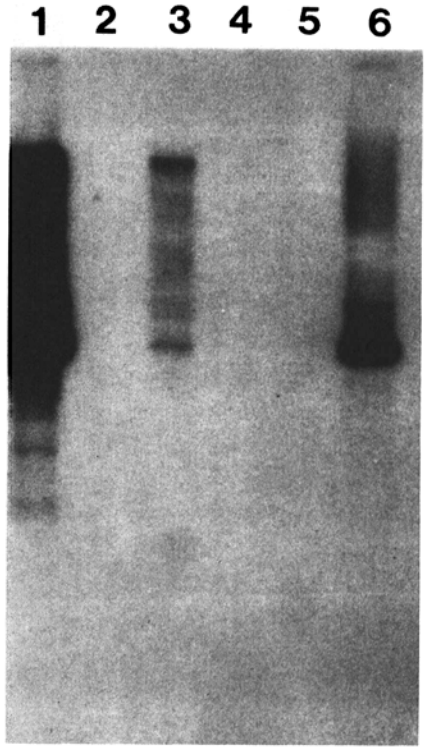

Fig. 2. Autoradiograph of Southern blot hybridization of ^{32}P-labeled potato leafroll luteovirus (PLRV) cDNA to reverse transcription-polymerase chain reaction (RT-PCR) amplified cDNA products of PLRV and strawberry mild yellow-edge luteovirus (SMYEV). Total nucleic acids of PLRV-infected and uninfected potato leaf tissue (lanes 1 and 2, respectively); minicolumn-purified total nucleic acids of SMYEV-infected and uninfected strawberry leaf tissue (lanes 3 and 4, respectively); total nucleic acids of SMYEV-infected strawberry leaf tissue before minicolumn purification (lane 5); and purified PLRV RNA (lane 6).

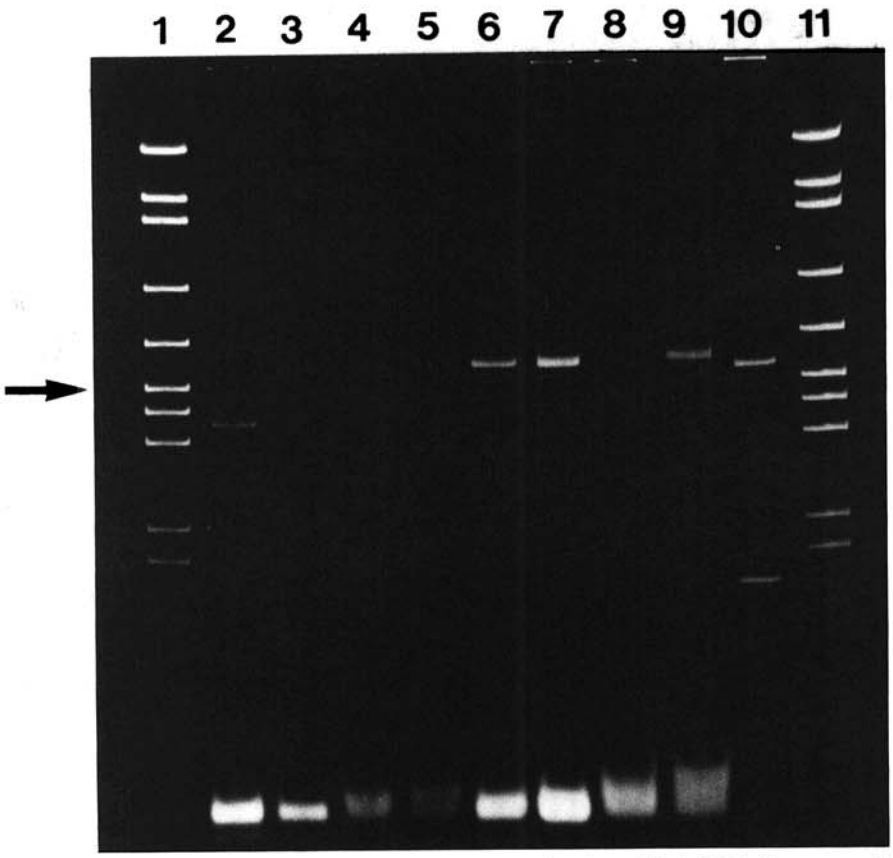

Fig. 3. Restriction fragment length polymorphism analysis of amplified cDNA fragments of potato leafroll luteovirus (PLRV) and strawberry mild yellow-edge luteovirus (SMYEV). Fragments were digested with restriction endonucleases *Sau*3AI or *Sin*I. pGEM DNA markers, arrow indicates 460 bp (lanes 1 and 11); *Sau*3AI-digested PLRV cDNA and SMYEV cDNA (lanes 4 and 8, respectively); *Sin*I-digested PLRV cDNA (lanes 2 and 5); PLRV cDNA control (lanes 3 and 10); *Sin*I-digested SMYEV cDNA (lanes 6 and 9); and SMYEV cDNA control (lane 7).

PCR amplified products observed for PLRV in the presence of the enzyme were not detected when it was omitted from the assay (*results not shown*). Thus the infection-associated DNA appears to be a host contaminant, because reverse transcription of PLRV RNA to cDNA is required for its amplification by PCR.

To test the utility of the RT-PCR assay method for routine detection of PLRV, leaf samples were collected from potato plants grown under natural field conditions at the U.S. Department of Agriculture facility in Presque Isle, Maine. Fig. 5 shows polyacrylamide gel electrophoretic analysis of the RT-PCR amplified products of field samples (lanes 4–14) and control samples (lanes 1–3). Field samples with an amplified cDNA fragment of 487 bp were considered positive for PLRV (lanes 5–8 and 10–14). Two samples were considered negative for PLRV, because the 487-bp cDNA was not detected (lanes 4 and 9).

Sensitivity of PLRV RNA detection by RT-PCR. To determine the sensitivity of RT-PCR, different amounts of purified PLRV RNA were reverse-transcribed to synthesize PLRV cDNA. Transcripts were then amplified, and RT-PCR products were analyzed by gel electrophoresis. The limit of PLRV RNA detection by RT-PCR assay was 100 fg of RNA in the RT reaction based on the presence of the 487-bp product (Fig. 6, lane 5). In similar experiments, the limit of PLRV RNA detection in total nucleic acids of infected potato leaves by RT-PCR assay was 30 pg of total nucleic

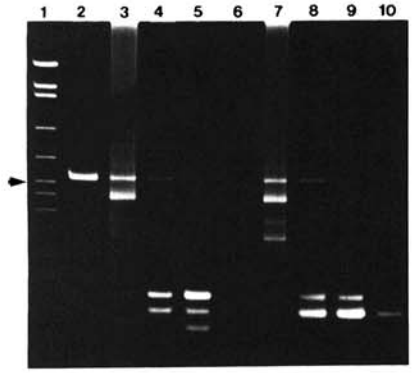

Fig. 4. Polyacrylamide gel electrophoretic analysis of reverse transcription-polymerase chain reaction (RT-PCR) amplified potato leafroll luteovirus (PLRV) cDNA in total nucleic acids from different tissues of PLRV-infected potato tubers. pGEM DNA markers (lane 1—arrow indicates 460 bp); RT-PCR products of purified PLRV RNA (lane 2); RT-PCR products of PLRV-infected potato tuber tissue enriched in: periderm and cortex (lanes 3 and 7), "eye" bud (lanes 4 and 8), cortex and vascular ring (lane 5), perimedullary zone (lane 9), and medulla (pith) (lanes 6 and 10). Three μg of total nucleic acids for each sample was reverse transcribed and amplified as described in Materials and Methods. Five μl of amplified DNA/sample was analyzed.

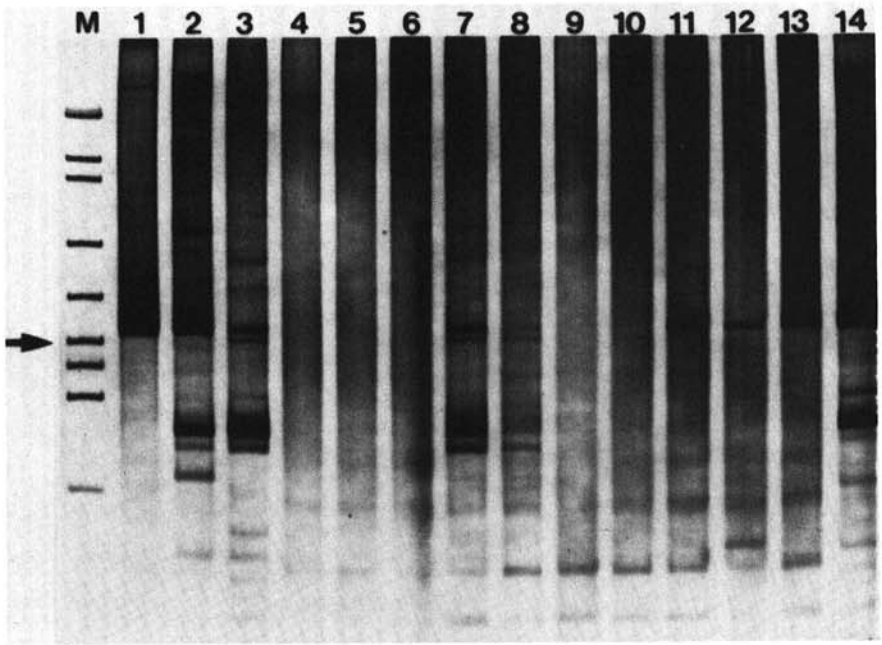

Fig. 5. Polyacrylamide gel electrophoretic analysis of reverse transcription-polymerase chain reaction (RT-PCR) products amplified from total nucleic acids of leaf samples collected from a potato field. pGEM DNA markers (lane M—arrow indicates 460 bp); purified potato leafroll luteovirus (PLRV) RNA control (lane 1); PLRV-infected potato leaf control (lane 2); and potato leaf field samples (lanes 3–14). Polyacrylamide gel was stained with silver nitrate.

acids in the RT reaction (*results not shown*).

Detection of PLRV RNA in viruliferous aphids by RT-PCR. Polyacrylamide gel electrophoretic and Southern blot hybridization analyses of RT-PCR amplified PLRV cDNA fragments of total nucleic acids from viruliferous and nonviruliferous aphids were compared with the analyses of RNA from purified PLRV and total nucleic acids from PLRV-infected potato leaves (Fig. 7A and B). A major cDNA product of 487 bp from viruliferous aphids (Fig. 7A, lanes 3 and 4), identical in size to that obtained from viral RNA and infected plant tissue (Fig. 7A, lanes 1 and 2, respectively), hybridized with a ^{32}P-labeled PLRV cDNA probe of the viral coat protein gene (Fig. 7B, lanes 3 and 4). A DNA fragment of approximately 1,200 bp that hybridized with the labeled probe was also observed as a minor component in samples of the viruliferous aphids (Fig. 7A and B, lanes 3 and 4). No major amplified product or hybridization was obtained with samples of nonviruliferous aphids (Fig. 7A and B, lane 5). Amplified PLRV cDNA products of viruliferous aphids that fed on PLRV-infected potato or *D. stramonium* gave similar electrophoretic and hybridization patterns (Fig. 7A and B, lanes 3 and 4). These patterns were different from that of infected leaf tissue, because a minor cDNA fragment of approximately 700 bp from the infected tissue was amplified and hybridized with the probe (Fig. 7A and B, lane 2). Vector-specific cDNA products, less than 487 bp, were observed with viruliferous and nonviruliferous aphids (Fig. 7A, lanes 3–5). These products did not hybridize with the PLRV cDNA probe (Fig. 7B, lanes 3–5).

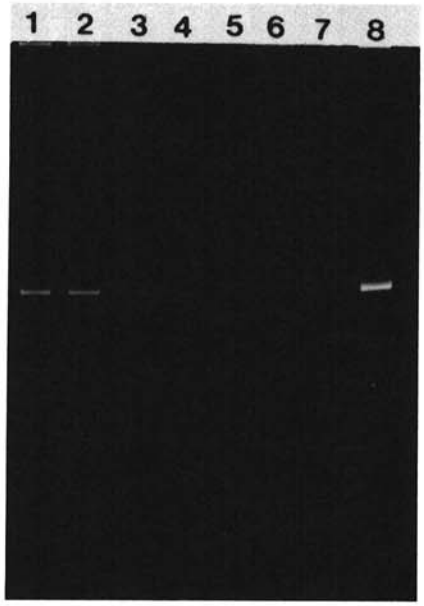

Fig. 6. Polyacrylamide gel electrophoretic analysis of reverse transcription-polymerase chain reaction (RT-PCR) amplified potato leafroll luteovirus (PLRV) cDNA products synthesized from different amounts of purified PLRV RNA. Amplified PLRV cDNA products from the following amounts of purified PLRV RNA in the RT reactions were analyzed: 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and total nucleic acids (3 μg) of PLRV-infected potato leaf tissue control. Samples were reverse transcribed, 0.1 volume of each reaction mixture was amplified by PCR in a total volume of 50 μl, and 5 μl of each amplified product was analyzed on polyacrylamide gels. Therefore, the equivalent amount of purified PLRV RNA analyzed on polyacrylamide gels was 10 pg (lane 1), 1 pg (lane 2), 100 fg (lane 3), 10 fg (lane 4), 1 fg (lane 5), 0.1 fg (lane 6), 0.01 fg (lane 7), and total nucleic acids (30 ng) of PLRV-infected potato leaf tissue control. Samples were visualized by ethidium bromide staining.

DISCUSSION

This study demonstrated the successful use of RT-PCR to directly detect luteoviruses from PLRV RNA in total nucleic acids of infected potato leaves and tubers and in viruliferous aphids, and SMYEV RNA in nucleic acid extracts of infected strawberry leaves. Our study also shows the feasibility of RT-PCR as a rapid laboratory assay for detecting PLRV from field samples. From 3 to 5 days were required for positive identification of the virus from infected potato tissue or viruliferous aphids. The availability of the nucleotide sequence of the coat protein gene of PLRV enabled us to design a primer pair that specifically amplified, in RT-PCR reactions, a 487-bp PLRV cDNA fragment covering most (77%) of the coat protein region of PLRV RNA, and an approximately 500-bp SMYEV cDNA fragment. The specificity of the 487-bp DNA fragment amplified from PLRV RNA was based on the following: 1) reverse transcriptase was required for its synthesis; 2) the fragment, which hybridized specifically with ^{32}P-labeled PLRV cDNA of the coat protein gene, was detected in total nucleic acids of both PLRV-infected plant tissue and viruliferous aphids; and 3) the fragment was absent from total nucleic acids of uninfected potato tissue and nonviruliferous aphids. The dependence of PCR on reverse transcription of PLRV RNA to cDNA indicates that the DNA reported to be associated with purified virions (26,37) is a host contaminant and not an intermediate in viral genome and/or coat protein synthesis. The specificity of the SMYEV cDNA fragment was based on the following: 1) the size of the major cDNA fragment from SMYEV-infected strawberry tissue was slightly larger than the 487 bp of PLRV; 2) in specific cross hybridization with the ^{32}P-labeled cDNA probe of the coat protein gene of PLRV, heterologous hybridization signals were less intense than those of homologous hybridization; 3) hybrid-

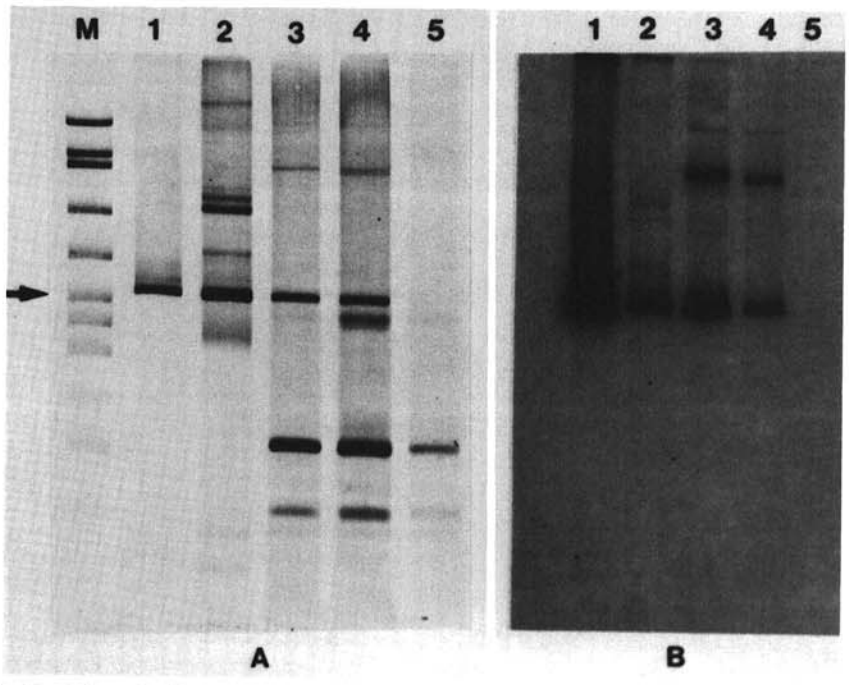

Fig. 7. Polyacrylamide gel electrophoretic analysis **(A)** and autoradiograph of Southern blot hybridization analysis **(B)** of reverse transcription-polymerase chain reaction (RT-PCR) products from viruliferous and nonviruliferous aphids. pGEM DNA markers (lane M—arrow indicates 460 bp); purified potato leafroll luteovirus (PLRV) RNA control (lane 1), PLRV-infected potato leaf control (lane 2), viruliferous aphids maintained on PLRV-infected potato and *Datura stramonium* plants (lanes 3 and 4, respectively), and nonviruliferous aphids maintained on tobacco plants (lane 5). Polyacrylamide gel was stained with silver; Southern blot was hybridized with a ^{32}P-labeled PLRV cDNA probe specific for PLRV coat protein gene. RT-PCR products of nonviruliferous and viruliferous aphids were obtained from approximately one insect equivalent.

ized cDNA fragments were absent from uninfected strawberry tissue; and 4) RFLP analysis revealed the fragment's similarity to PLRV cDNA when digested with *Sau*3AI, and its difference when digested with *Sin*I. These results suggest that a luteovirus is associated with SMYE disease and that the virus is distinct from PLRV. These findings affirm previous studies that suggest a luteovirus as a possible etiological agent of SMYE disease in Oregon (3,27,38) and Japan (47). Our results also suggest that size, RFLP, and hybridization analyses used to differentiate between PLRV and SMYEV could be used to distinguish between PLRV and any other luteovirus that may co-infect potato plants and/or be co-transmitted by viruliferious aphids.

Robertson et al (34) used luteovirus group-specific degenerate short primers (14–15 mers) to amplify a 530-bp cDNA from PLRV-infected *Physalis floridana* Rydb. In contrast, we have used non-degenerate primers (22 mers) based on the nucleotide sequence of the coat protein gene of PLRV to amplify and detect a 487-bp PLRV cDNA from PLRV-infected potato leaves and tubers, and from viruliferous aphids. Our detection of the 487-bp major DNA fragment indicated the successful isolation of an undegraded PLRV RNA fragment containing the coat protein gene from infected potato plant tissue and viruliferous aphids, and the specific priming of an efficient reverse transcription reaction. The amplification of the minor 700-bp and 1,200-bp DNA fragments from infected plant tissue and viruliferous aphids, respectively, suggests a difference(s) in the quality of isolated PLRV RNA, in the mechanisms of PLRV RNA processing in plants and insects, and/or in the RT-PCR reactions.

In addition to the above PLRV cDNA fragments, host- and vector-specific PCR products were observed with PLRV primers. Changing the parameters of RT-PCR reaction conditions did not abolish these products. Host-specific PCR products amplified with luteovirus group-specific degenerate primers have also been reported (34).

Gel electrophoretic analysis of the RT-PCR amplified products indicates that RT-PCR is potentially useful for routine PLRV diagnosis in potato tuber and leaf field samples. Current methods for detecting PLRV depend on aphid transmission to indicator hosts, enzyme-linked immunosorbent assay (ELISA), and/or molecular probes. The calculated theoretical minimum amount of purified PLRV RNA required for detection by RT-PCR amplification and polyacrylamide gel electrophoretic analysis is 1 fg of RNA, which corresponds to about 3 fg of virus particles. This is about 10^4-fold and 10^3-fold more sensitive than molecular hybridization with ^{32}P-labeled cDNA and cRNA probes, respectively (35); and about 10^4-fold more sensitive than detection by ELISA (41). This increase in sensitivity should permit PLRV infections in potato crops to be detected earlier than is now possible. The sensitivity of RT-PCR might be increased when polyacrylamide gels containing the amplified DNA products are analyzed by Southern blot hybridization rather than by staining. In view of its high sensitivity, the RT-PCR should also be valuable in epidemiological studies of PLRV, as well as in investigations of PLRV–vector–host interactions. Because of its sensitivity, the RT-PCR provides an alternative to the other diagnostic techniques without the need for large samples or molecular hybridization.

Recently, López-Moya et al (21) and Navot et al (31) reported the successful use of PCR in detecting the DNA viruses cauliflower mosaic and tomato yellow leaf curl from viruliferous aphids and whiteflies, respectively. Our study extends the application of PCR technology by demonstrating the detection of viral RNA in vectors which transmit viruses in a circulative persistent manner. To our knowledge, PLRV is the first RNA virus definitively detected from its vector by RT-PCR.

At present, a luteovirus and a potexvirus have been implicated in the etiology of SMYE disease. It is possible that these two viruses form a complex which results in the disease, and/or that the two viruses could cause two indistinguishable diseases. In each case, the geographical distribution of the virus may depend on its prevalence in strawberry germ plasm and the distribution of aphid vectors in the area. The elucidation of the vector association and the disease-causing interactions of this unusual luteovirus–potexvirus complex by RT-PCR technology will require additional studies.

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