Detection of Potato Leafroll Virus in Leaf and Aphid Extracts by Dot-Blot Hybridization

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ABSTRACT

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Cloned cDNA to potato leafroll virus (PLRV) RNA was evaluated for detection of PLRV in virus-infected samples from four states and the countries of Colombia and Russia. The use of formaldehyde was 32 times more effective than formamide for denaturing leaf tissue extracts in the dot-blot assay, and the sensitivity of the technique was estimated to equal that of the enzyme-linked immunosorbent assay (ELISA) for PLRV detection. These results, use of a commercial leaf sap extractor, detection of virus in bulk aphid samples, and use of the assay in a nonradioactive format demonstrate that the technology has potential for large-scale disease screening programs.

Potato leafroll virus (PLRV) is a luteovirus (10) and an important aphid-transmitted virus of potato (16,17). The isometric particles (25 nm diameter) of PLRV are confined to phloem tissue of infected plants and are not transmitted by sap inoculation (13). Virions of PLRV consist of a major coat protein component of 26 kDa (19) and a positive-sense, single-stranded RNA of 6 kb (23).

Host symptomatology, aphid transmissions to indicator plants, and tests for callose deposition in phloem have been used to diagnose potato leaf roll (PLR) or detect PLRV (5). The enzyme-linked immunosorbent assay (ELISA) has been widely used to detect PLRV coat protein in infected plants since 1977 (6,21).

More recently, a variety of plant viruses (4,9,11,14,20,27) and viroids (3,15) have been detected by nucleic acid hybridization techniques. We investigated the use of this technique to detect PLRV and showed that PLRV is readily detected in leaf and aphid extracts by cDNA/RNA dot-blot hybridization technology. In addition, we found that formaldehyde is a more effective denaturant than formamide for sample denaturation, increasing assay sensitivity by an estimated factor of 32 times.

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MATERIALS AND METHODS

Virus isolate and cloned cDNA. PLRV isolate 4 (PLRV4) and its associated cDNA plasmid clones pPLRV4-173, -228, and -323 have been described (24). Potato (*Solanum tuberosum* L. 'Russet Burbank') leaves from plants grown from PLRV4-infected seed tubers were used as positive controls. Leaves from non-infected plants (PLRV-free) served as negative controls.

Virus samples. Potato leaf samples infected with domestic isolates of PLRV were from greenhouse-grown PLRV-infected seed tubers shipped from cooperators. Samples from Idaho, Maine, Washington, and Wisconsin were provided by D. L. Corsini, F. E. Manzer, P. E. Thomas, and S. A. Slack, respectively. Leaf samples infected with foreign isolates of PLRV were obtained from the National Plant Germplasm Quarantine Laboratory, USDA-ARS, Glenn Dale, Maryland. Samples of PLRV-exposed green peach aphids, *Myzus persicae* (Sulzer), were from a colony reared continuously on *Physalis floridana* Rydb. infected with PLRV4 (24). PLRV-free aphids were from a colony reared on turnip (*Raphanus sativus* L.) which is immune to PLRV (8).

Preparation of leaf tissue. Leaves (2–3 g fresh weight) were pulverized in liquid nitrogen and homogenized with 3 volumes of 50 mM sodium phosphate buffer, pH 7.0, for 45 sec using a Tissuemizer (Tekmar Co., Cincinnati, OH). The homogenate was centrifuged (3,000 *g*) at 4 C for 15 min and filtered through cheesecloth to remove leaf debris, and the supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Equal volumes of the clarified supernatant were denatured with formamide or formaldehyde to directly compare the two denaturants.

Preparation of leaf sap. Leaf sap was expressed using a Model 1 Leaf Squeezer (Ravenel Specialities Co., Seneca, SC) and 250 μl was transferred to a 1.5-ml microfuge tube. After the addition of 750 μl of 50 mM sodium phosphate buffer, pH 7.0, cell debris was removed by centrifugation (14,000 *g*) at 4 C for 5 min. The supernatant was extracted as described for leaf tissue and processed for formaldehyde sample denaturation.

Preparation of aphids. Test samples comprised of 20 aphids each were transferred to 1.5-ml microfuge tubes and homogenized with 100 μl of 50 mM sodium phosphate buffer, pH 7.0, and 100 μl of phenol/chloroform/isoamyl alcohol (25:24:1) for 1 min using a handheld micropestle. After centrifugation (14,000 *g*) at 4 C for 10 min, the clarified supernatant was processed for formaldehyde sample denaturation. The proportion of virus-exposed aphids in each sample ranged from 0/20 to 20/20.

Formamide sample denaturation. Samples were processed following the methods of Garger et al (11). Briefly, 1 volume of clarified supernatant was combined with 1 volume of 100% formamide, incubated at 100 C for 3 min, then cooled on ice and stored at −70 C. Just prior to placing on nitrocellulose membranes, thawed samples were adjusted to 10× SSC by the addition of an equal volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0).

Formaldehyde sample denaturation. One volume of clarified supernatant was combined with 3 volumes of 20× SSC:37.3% formaldehyde (1:1), incubated at 65 C for 15 min, then cooled on ice and stored at −70 C (24).

Dot-blot hybridization. Nitrocellulose membranes (BA 85, 0.45 μm, Schleicher & Schuell, Keene, NH) were treated with 10× SSC (10 min) before use, and wells of a Model 1 multi-well vacuum filtration manifold (Schleicher & Schuell) were washed with 10× SSC (400 μl) before sample application. After sample (400 μl) application, wells were washed with 10× SSC (400 μl). Membranes were then air-dried (1 hr) and baked in vacuo (80 C) for 2 hr. Processing of the blots for prehybridization (4 hr), hybridization (20 hr), washing, and autoradiography (48 hr) were as described (24). For aphid samples, 100 μg/ml of yeast tRNA, 10 μg/ml of poly(A), and 10 μg/ml of poly(C) (2) were added to the prehybridization and hybridization solutions. Clones pPLRV4-173, -228, and -323 were prepared as ^{32}P-labeled hybridization probes by nick translation (18).

Processing of leaf tissue samples for analysis by ELISA and dot-blot hybridization. Leaf tissue from a PLRV4-infected Russet Burbank plant was pulverized in liquid nitrogen, weighed, and divided into two samples of 2 g each. One sample was processed for dot-blot hybridization using 3 volumes of 50 mM sodium phosphate buffer, pH 7.0, and formaldehyde, as described above. The other sample was processed for ELISA using 3 volumes of extraction buffer (phosphate-buffered saline [PBS], pH 7.4, [12] containing 0.2% nonfat dried milk and 0.05% Tween 20). Both samples were homogenized and clarified by centrifugation and cheesecloth filtration (see above). A 100 μl extract for ELISA was similar to a 400 μl dot-blot extract on a tissue equivalent basis. ELISA was conducted with 100-μl volumes (paired wells) in a direct double-sandwich procedure (7) that employed commercially available PLRV IgG and alkaline phosphatase-conjugated PLRV IgG (Agdia, Inc., Mishawaka, IN). Plates were coated with 2 μg/ml of coating IgG, incubated, washed with PBS containing 0.05% Tween 20, incubated with the sample, washed, and incubated with a 1:1,000 dilution (in extraction buffer) of alkaline phosphatase-conjugated IgG. All incubations were overnight at 4 C. Following the last incubation and washing, substrate was added; and 1 hr after the addition, the reactions were read at 405 nm with a Molecular Devices Vmax Reader. For dilution endpoint comparisons, ELISA samples were serially diluted with extraction buffer, and dot-blot samples were serially diluted with 7.5× SSC, 14% formaldehyde.

Testing of samples infected with two other potato viruses. Potato leaf samples infected with potato virus X (PVX), potato virus Y (PVY), and PLRV were provided by R. W. Goth (Vegetable Laboratory, USDA-ARS, Beltsville, MD) and included confirmation by ELISA testing. Virus-free samples were provided as controls. Pulverized leaf tissue was extracted, denatured with formaldehyde, and processed for radioactive dot-blot hybridization as described above. Nonradioactive hybridization was performed on a replicate nitrocellulose membrane using a commercially available digoxigenin-based DNA labeling and immunodetection kit (Cat. No. 1093 657 Boerhinger Mannheim Biochemicals, Indianapolis, IN). The prehybridization (4 hr) and hybridization steps (20 hr) included 50% formamide and were conducted at 42 C using the solutions recommended by the manufacturer. The hybridization step used 25 ng/ml of digoxigenin-labeled DNA followed by posthybridization washing as described (24). Detection of digoxigenin-hybridized DNA with anti-digoxigenin-alkaline phos-

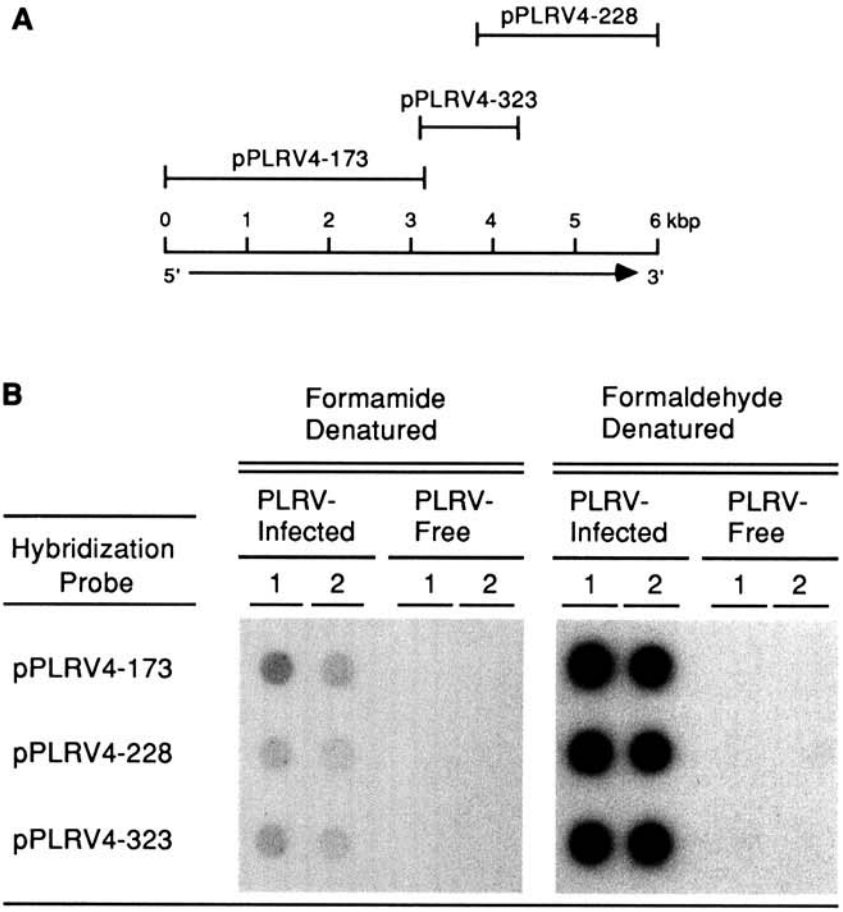

Fig. 1. Detection of potato leafroll virus (PLRV) isolate 4 (PLRV4) in extracts from pulverized leaf tissue using dot-blot hybridization and PLRV4 cDNA probes. **(A)** Location of the PLRV4 cDNA probes relative to the viral RNA genome (24). **(B)** Dot-blot hybridization using nick-translated, ^{32}P-labeled probes. Two PLRV4-infected and PLRV-free Russet Burbank plants were processed for denaturation using formamide or formaldehyde.

phatase conjugate followed the manufacturer's instructions and used a 3-hr substrate reaction time.

RESULTS

The cDNA clones pPLRV4-173, -228, and -323 produced in an earlier study (24) readily hybridized to viral RNA in extracts from PLRV4-infected leaf tissue. As shown in Figure 1, the autoradiographic signals produced by formaldehyde-denatured samples were greater than parallel samples treated with formamide. Hybridization of these probes to extracts of PLRV-free leaves was not observed (Fig. 1).

Because pPLRV4-173 does not hybridize to the coat protein coding region of the viral genome (23), it was selected for use in subsequent experiments. Figure 2 shows the results of testing pPLRV4-173 against isolates of PLRV from the United States and shows that this probe hybridized to PLRV-infected samples from Idaho, Maine, Washington, and Wisconsin. Figure 2 also shows that formaldehyde-denatured samples produced greater autoradiographic signals than did formamide-denatured samples, confirming the results presented in Figure 1.

To estimate the increase in sensitivity associated with formaldehyde denaturation, two PLRV-infected Russet Burbank leaf tissue samples, one each from Idaho and Wisconsin, were tested. Figure 3 shows the results of using a twofold dilution series to compare formamide to formaldehyde for sample denaturation. Analysis of the Wisconsin sample (Fig. 3, sample 1) shows that the dilution endpoint produced by formaldehyde denaturation (1:128) was 32-fold greater than formamide denaturation (1:4). Analysis of the Idaho sample (Fig. 3, sample 2) paralleled this result.

To directly compare the sensitivity of dot-blot hybridization (formaldehyde denaturation) to ELISA for virus detection, leaf tissue from a PLRV4-infected Russet Burbank plant was processed for virus detection by both assays. As shown in Figure 4, the dilution endpoint determined for both assays was equal, corresponding to 1:32.

The results of using squeezed leaf sap for virus detection by dot-blot hybridization are shown in Figure 5. Virus was readily detected in the leaf sap of PLRV-infected samples from the countries of Russia and Colombia. Hybridization to sap extracts of PLRV-free leaves was not observed.

Dot-blot hybridization of bulk aphid samples containing various proportions (0/20 to 20/20) of virus-exposed aphids is presented in Figure 6. The intensities of autoradiographic signals increased with increases in the proportion of virus-exposed aphids in the samples. The signals produced by larger proportions of virus-exposed aphids were intense and positive. The signals from samples containing one (1/20) or two (2/20) virus-exposed aphids emitted faint autoradiographic signals which scored weakly positive in relation to the signal produced by virus-free aphids samples (0/20).

Testing of the viral specificity of the assay using two common potato viruses was also investigated, including a nonradioactive format. As shown in Figure 7, hybridization to leaf extracts from PVX- or PVY-infected potato plants was not observed. These results confirm the viral specificity of the assay and demonstrate that PLRV is detectable by nonradioactive dot-blot hybridization (Fig. 7B).

DISCUSSION

Since the first report by Owens and Diener (15) on the application of cDNA/

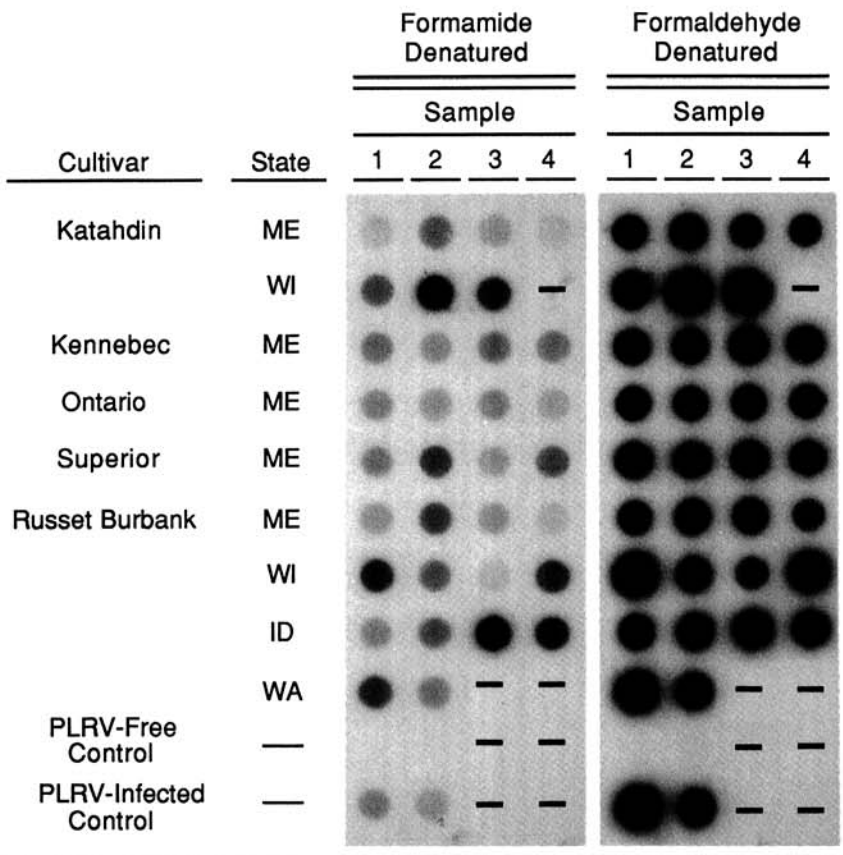

Fig. 2. Detection of potato leafroll virus (PLRV) in domestic samples of virus-infected potato plants by dot-blot hybridization. The hybridization was conducted using a 5′ viral cDNA probe, pPLRV4-173 (Fig. 1). Leaf tissue samples were pulverized and processed for denaturation using formamide or formaldehyde. State abbreviations are Idaho (ID), Maine (ME), Washington (WA), and Wisconsin (WI). Two to four independent samples from each state were processed.

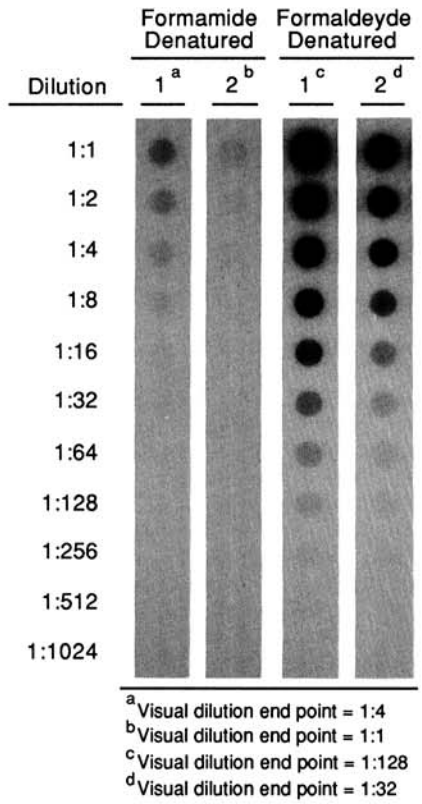

Fig. 3. Dilution endpoint comparison of formamide versus formaldehyde as denaturants for the detection of potato leafroll virus (PLRV) by dot-blot hybridization. Samples 1 and 2 are PLRV-infected Russet Burbank pulverized leaf tissue samples from Wisconsin and Idaho, respectively. The hybridization was conducted using a 5′ viral cDNA probe, pPLRV4-173 (Fig. 1). For both samples, the dilution endpoint for formaldehyde denaturation exceeded formamide denaturation 32-fold. Samples were homogenized with buffer (1:3) and clarified prior to conducting the twofold dilution series.

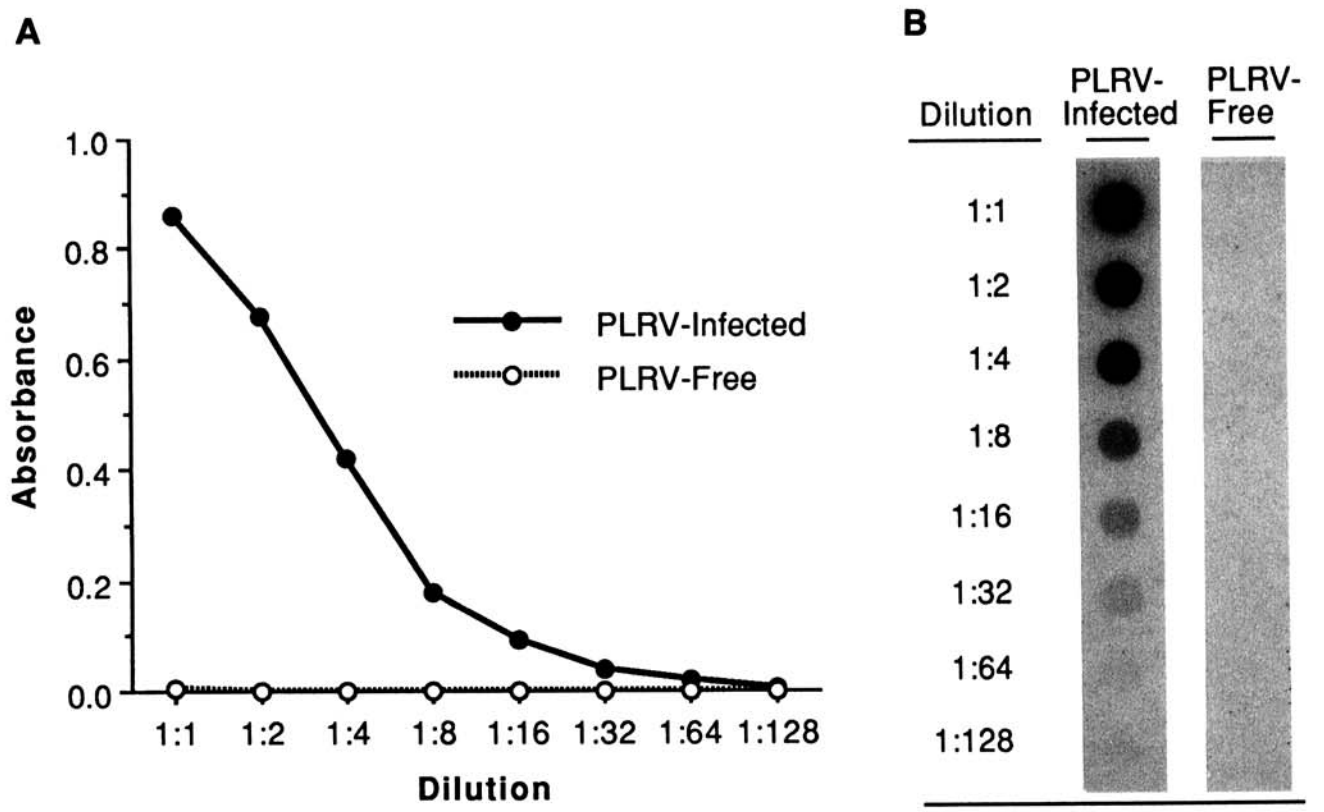

Fig. 4. Comparison of dot-blot hybridization and enzyme-linked immunosorbent assay (ELISA) for potato leafroll virus (PLRV) detection using a twofold dilution series. **(A)** ELISA. **(B)** Dot-blot hybridization (formaldehyde denaturation). PLRV4-infected and noninfected (PLRV-free) Russet Burbank pulverized leaf tissue samples were homogenized with buffer (1:3) and clarified prior to conducting the twofold dilution series. Absorbance values for ELISA are the average of two wells. The hybridization was conducted using a 5′ viral cDNA probe, pPLRV4-173 (Fig. 1). The dilution endpoint for both assays corresponded to 1:32.

RNA dot-blot hybridization to the detection of an RNA pathogen, potato spindle tuber viroid, the technology has been successfully applied to a wide variety of plant RNA viruses and other viroids.

The binding (dot-blotting) of RNA to nitrocellulose is dependent on treatment of the nitrocellulose with high salt (25) and is further improved by the denaturation of RNA (26). White and Bancroft (28) were first to report the use of formaldehyde-denatured samples in dot-blot hybridizations. These investigators monitored prolactin mRNA expression in cytoplasmic preparations of animal samples using cDNA/RNA dot-blot hybridization, and demonstrated that formaldehyde was more effective than glyoxal for sample denaturation. Although formamide (11) and formaldehyde (20) have been used as denaturants for the detection of RNA plant viruses by dot-blot hybridization, a comparison of these two denaturants on assay sensitivity has not been reported. Our direct comparison of formamide to formaldehyde shows that the latter increases assay sensitivity 32-fold, at least with respect to PLRV detection (Fig. 3).

The fact that cloned cDNA (pPLRV4-173) to PLRV4 hybridized to samples of PLRV from four states (Fig. 2) and the countries of Colombia and Russia

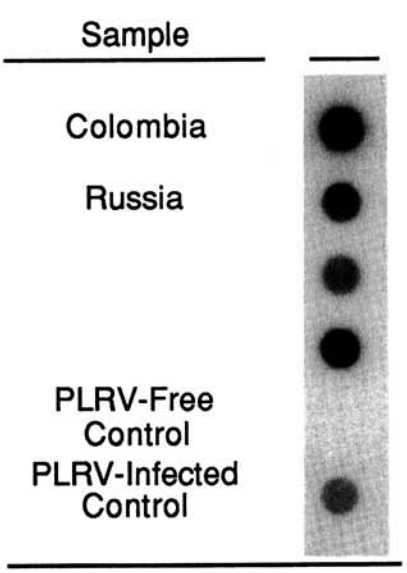

Fig. 5. Detection of potato leafroll virus (PLRV) by dot-blot hybridization using samples prepared with a commercial leaf extractor. Leaf sap of PLRV-infected potato plants from Colombia (n = 1) and Russia (n = 3) was expressed using a commercial leaf squeezer. Controls consisted of leaf sap squeezed from PLRV-free potato leaves and ones infected with PLRV isolate 4. Clarified sap extracts were denatured with formaldehyde and tested using a 5′ viral cDNA probe, pPLRV4-173 (Fig. 1).

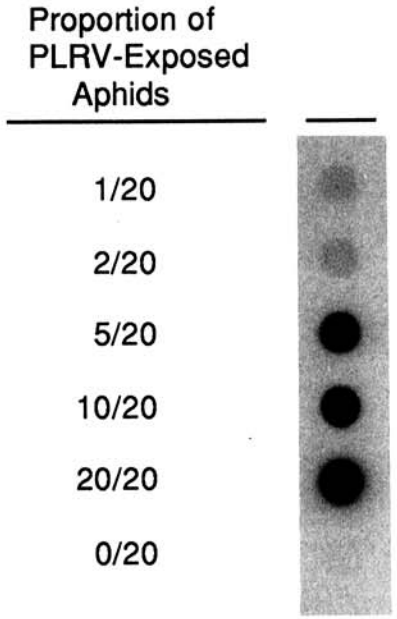

Fig. 6. Detection of potato leafroll virus (PLRV) in virus-exposed green peach aphids by dot-blot hybridization. Samples (20 aphids/sample) containing various proportions (0/20 to 20/20) of virus-exposed aphids were denatured with formaldehyde and hybridized using a 5′ viral cDNA probe, pPLRV4-173 (Fig. 1). Virus-exposed aphids were from a colony reared continuously on *Physalis floridana* Rydb. infected with PLRV isolate 4.

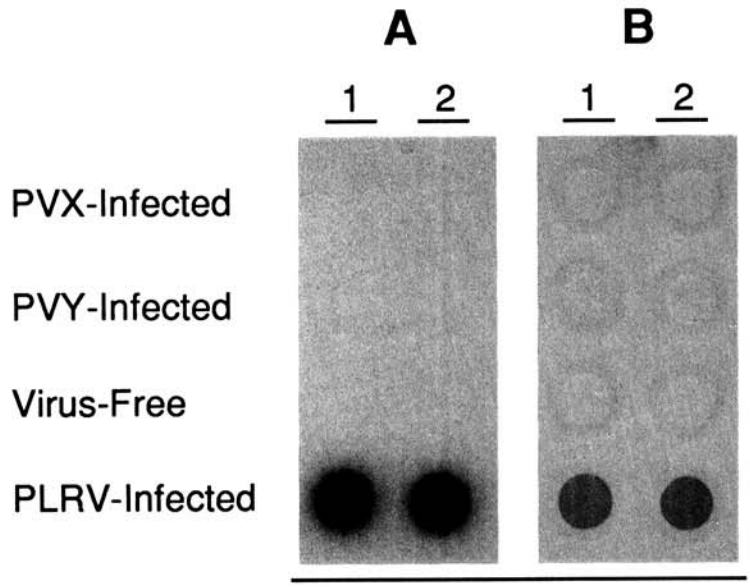

Fig. 7. Confirmation of the viral specificity of the dot-blot hybridization assay using two common potato viruses. Replicate nitrocellulose membranes were processed for radioactive **(A)** and nonradioactive **(B)** dot-blot hybridization. Two independent plants infected with potato virus X (PVX) or potato virus Y (PVY) were assayed. Potato leafroll virus (PLRV) infected and virus-free plants were processed as positive and negative controls, respectively. Pulverized leaf tissue extracts from potato were denatured with formaldehyde and tested using a 5′ viral cDNA probe, pPLRV4-173 (Fig. 1).

(Fig. 5) illustrates the assay's ability to detect geographically diverse isolates. These results, the preparation of samples using a commercial leaf sap extractor (Fig. 5), the detection of virus in bulk aphid samples (Fig. 6), and the successful use of the assay in a nonradioactive format (Fig. 7) all suggest that dot-blot hybridization technology has potential for large-scale disease screening programs.

Dot-blot hybridization is effective for the diagnosis of PLR and supplements existing disease detection assays (ELISA and aphid-indexing to indicator plants) with one based on viral RNA and cDNA technology. ELISA is dependent on the viral coat protein, which has been mapped to the 3′ half of the PLRV RNA genome (23). In contrast, using the dot-blot hybridization assay, it is possible to assay any portion of the viral genome by choosing from an available assortment of cloned and mapped PLRV cDNAs (23,24). The 5′ probe pPLRV4-173 (Fig. 1), for example, could be used to monitor virus infection in plants that have been genetically engineered for disease resistance based on the expression of the viral coat protein gene (1,22). Dot-blot hybridization might also be useful in elucidating virus–vector interactions. Sequential, time-lapse, dot-blot hybridizations of aphids following PLRV acquisition might resolve the question of whether or not PLRV replicates in its vector (13).

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