Hybridization Detection of Insect-Transmitted Plant Viruses with Digoxigenin-Labeled Probes

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ABSTRACT

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A dot blot hybridization system using digoxigenin-labeled probes and colorimetric visualization was developed for detection of plant viruses. Geminiviruses, including squash leaf curl virus and beet curly top virus, as well as zucchini yellow mosaic potyvirus, lettuce infectious yellows virus, and beet yellows closterovirus, were detected using this system. Extraction of the viral nucleic acid was necessary for effective detection of the viruses. Although the colorimetric system was less sensitive than radioactive dot blot hybridization, it was adequate for the detection of viruses from infected plants. This colorimetric system can be carried out in diagnostic laboratories using minimal equipment, suggesting its applicability for use in routine plant virus diagnoses.

Additional keyword: nonradioactive

Although serological methods are often used in routine detection of viruses from plant tissues, nucleic acid hybridization allows for more specific and sensitive testing. The A component DNA of a specific geminivirus can be used as a general hybridization probe to detect most whitefly-transmitted geminiviruses, whereas a probe derived from DNA B of the same geminivirus can be used to specifically identify that virus (6). Dot blot procedures have been developed that allow rapid preparation of plant samples for hybridization-based detection, and nucleic acid hybridization with cloned probes is commonly used for detection of whitefly-transmitted geminiviruses (1,4,9), as well as a range of additional plant viruses (8).

Radioactively labeled probes have been commonly employed for nucleic acid hybridization. However, concerns about the environmental impact, safety, and cost of using radioactive labels have prompted the development of alternative hybridization methods that employ nonradioactive labels. The use of such hybridization methods for detection of plant viruses has increased in recent years, with digoxigenin (dig)-labeled probes being used with a select few viruses (2,3,5,7). Unfortunately, the sample preparation methods used for each virus are specific for the virus or host tissue and are not well adapted for a range of unknown viruses. This paper presents extraction, probe preparation, and hybridization techniques for colorimetric detection of diverse plant viruses using a commercially available digoxigenin system.

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MATERIALS AND METHODS

Virus, vector source, and maintenance. Healthy insect colonies were reared under controlled temperature, light, and humidity. Sweetpotato whiteflies, *Bemisia tabaci*, were maintained on a mixture of bush snap bean, *Phaseolus vulgaris* "Top Crop" and bush lima bean "Fordhook." *Myzus persicae* aphids were reared on radish "Daikon," and *Circulifer tenellus* leafhoppers, originally provided by S. Trjapitzin (University of California, Riverside), were fed on sugar beet, *Beta vulgaris*.

Most viruses used for testing were maintained in greenhouse-grown herbaceous hosts and were transmitted regularly to fresh plants using the appropriate vector. Squash leaf curl geminivirus (SLCV), provided by J. E. Polston (University of Florida, Bradenton) and vectored by *B. tabaci*, was maintained on zucchini, *Cucurbita pepo*, "Black Beauty." Tomato mottle geminivirus-infected tomato was also provided by J. Polston. Beet curly top geminivirus (BCTV), provided by J. E. Duffus (USDA-ARS, Salinas, CA) and transmitted by *C. tenellus*, was maintained on sugar beet. Beet yellows closterovirus (BYV), collected from Stockton, CA, was vectored by *M. persicae* and was maintained in New Zealand Spinach, *Tetragonia expansa*. Lettuce infectious yellow virus (LIYV) was collected from lettuce in Imperial County, CA, transmitted by *B. tabaci*, and maintained on *Chenopodium murale*. Zucchini yellow mosaic potyvirus (ZYMV) was obtained from T. M. Perring (University of California, Riverside) and maintained in zucchini.

Sample preparation. The homogenization buffer used for extracting plant material for analysis consisted of 0.4 M Tris-HCl, pH 8.0; 1% sodium dodecyl sulfate (SDS); 5 mM EDTA, pH 8.0; and 4% β-mercaptoethanol (12). A solution consisting of 0.5 M sodium sulfite, 1% SDS, and 4% β-mercaptoethanol (13) was used for some geminivirus extractions.

Homogenates of plant tissue were prepared by one of several methods. Fresh or frozen tissue was ground in 1 ml of homogenization buffer per g of tissue with a chilled mortar and pestle. Alternatively, tissue was processed through a leaf squeezer (Model 1, Ravenel Specialties Co., Seneca, SC). The concentrated sap was directly denatured for dot blotting or diluted 1:1 in homogenization buffer for extraction.

Phenol extractions were performed by adding three volumes of 0.1 M Tris-HCl-saturated phenol (pH 8.0)/chloroform, 1:1, to 1 vol of buffered tissue homogenate, followed by vortexing. The aqueous phase was separated by centrifugation, collected, and extracted with an additional volume of phenol/chloroform, 1:1. In general, the aqueous phase was collected and denatured for dot blotting. Sometimes aqueous extracts were treated to remove residual phenol/chloroform by ethanol precipitation followed by resuspension in TE buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA).

One of two methods was employed to denature samples prior to dot blotting. For detection of geminiviruses, each sample was brought to 0.1 N hydroxide by the addition of 33 µl of 3 M sodium hydroxide per ml of homogenate or extract. After a 10-min incubation at room temperature, samples were neutralized by the addition of 0.1 ml of 3 M sodium acetate, pH 5.5, per ml of sample (9). Homogenates or extracts to be tested for RNA viruses were diluted 1:1 with 2× denaturation buffer (deionized 37% formaldehyde/20× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0] [11] [4:6]), then heated to 55°C for 15 min. Denatured homogenates were clarified by centrifuging 15,000 rpm for 5 min. This centrifugation was unnecessary for samples that had been extracted with phenol.

Samples were applied to nitrocellulose or positively charged nylon membranes at a rate of 25 µl per sample well under vacuum with a Bio-Dot blotting apparatus (Bio-Rad Laboratories, Richmond, CA). Blotted nitrocellulose membranes were baked at 80°C in a vacuum oven for 30 min; nylon membranes were baked for 30 min

at 120°C.

Some membranes blotted with unextracted plant homogenates were subjected to proteinase treatment just before baking the blots or immediately prior to hybridization of baked blots. Treatments were performed in 10-ml aliquots of proteinase reaction mixture in heat-sealable bags agitated for 1 to 3 h at 37°C. Proteinase K (Boehringer Mannheim, Mannheim, Germany; using high calcium conditions) and Pronase (Sigma Chemical Co., St. Louis) reactions were done as in Sambrook et al. (11).

Preparation and labeling of clones. SLCV A clone was obtained from J. A. Dodds (University of California, Riverside); a BCTV clone, pCFH-EB-3-1 (2.2-kb insert), was obtained from D. C. Stenger (Northern Illinois University, Dekalb). A cDNA clone to the BYV coat protein (620-bp insert) was obtained from V. V. Dolja (Oregon State University, Corvallis). A clone to ZYMV, pBA1 (2.8-kb insert), was obtained from A. Raffo (Columbia University, New York); an LIYV clone, pL105 (3-kb insert), was obtained from B. W. Falk (University of California, Davis).

Cesium chloride gradient-purified plasmids were used for labeling. Radioactive probes were labeled with (α-^{32}P)dCTP using nick translation. In preparation for labeling with digoxigenin, plasmids were either linearized or digested to excise the cloned cDNA completely. In general, plasmid fragments were not separated from probe DNA after digestion. However, when specific segments of cDNA were separated for labeling, the DNA was electrophoresed at 100 V for 1.5 h on 1% agarose gels (approximately 10 × 6.5 cm) in TAE buffer (0.04 M Tris-acetate, pH 8.0; 0.001 M EDTA) visualized by ethidium bromide staining, excised, and recovered using Geneclean (Bio 101, Inc., La Jolla, CA). Gel-isolated DNA was concentrated by ethanol precipitation. When digested plasmid and cloned DNA segments were not separated, the reaction mixture was extracted with phenol/chloroform, 1:1, and concentrated by ethanol precipitation.

Unlike radioactive probes, which can be efficiently generated by nick-translation incorporation of isotope, digoxigenin probes labeled by this method are much less sensitive than probes prepared by random priming. Incorporation of digoxigenin into DNA for use as probes was accomplished using random priming of DNA (11). Beginning with approximately 1 µg of template DNA, 50 to 250 ng of digoxigenin (dig)-labeled probe was obtained per reaction.

Hybridizations. Prehybridization solution for both dig-labeled and radioactive probes contained 50% deionized formamide in buffer (4× SSPE [1× SSPE is 0.15 M NaCl plus 0.01 M NaH_2PO_4 and 0.01 M EDTA, pH 7.4] [11] for radioactive probes; 5× SSC and 20 mM sodium maleate, pH 7, for dig-labeled probes), detergent (1% SDS or 0.02% SDS plus 0.1% *N*-lauroylsarcosine for radioactive or dig-labeled probes, respectively), and blocking reagents (0.5% nonfat powdered milk and 0.5 mg of denatured salmon sperm DNA per ml for radioactive probes or 2% blocking reagent [Boehringer] for dig-labeled probes). Prehybridizations were carried out at 42°C for 2 to 4 h, followed by replacement with fresh prehybridization mixture plus 100 to 200 ng of alkali-denatured (0.2 M NaOH) radioactive probes per ml or 5 to 25 ng of heat-denatured (100°C for 10 min) dig-labeled probe per ml for hybridization overnight at 42°C.

After hybridization, the dig-labeled hybridization solution was recovered and stored at –20°C for future use. Dig-labeled hybridization mixtures containing formamide were heated at 68°C for 10 min before reuse. Membranes hybridized with dig-labeled probes were washed twice in 2× SSC plus 0.1% SDS at room temperature for 15 min and twice in 0.5× SSC plus 0.1% SDS at 55 or 65°C for RNA or DNA viruses, respectively. ^{32}P-labeled blots were rinsed briefly in 2× SSC and washed at room temperature for 15 min each in 2×, 0.5×, and 0.1× SSC, all with 0.1% SDS, and at 55 or 65°C for 30 min in 0.1× SSC plus 1% SDS for RNA or DNA viruses, respectively. Washed membranes containing ^{32}P-hybridized blots were rinsed briefly in 0.1× SSC at room temperature, blotted gently, and exposed to film (Kodak X-Omat AR) for 1 to 3 days at –70°C with intensifying screens.

Detection of dig-labeled nucleic acids. Membranes were washed briefly in 150 mM NaCl and 100 mM Tris-HCl, pH 7.5, followed by incubation in the same buffer containing 20 mg of blocking reagent (Boehringer) per ml for 1 h. Blocked membranes were incubated for 1 h in anti-digoxigenin (Fab) antibody (Boehringer) conjugated to alkaline phosphatase diluted to 0.150 units per ml in blocking buffer. Immuno-reacted membranes were washed twice in 150 mM NaCl and 100 mM Tris-HCl, pH 7.5, for 15 min and equilibrated in 100 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5, for 5 min.

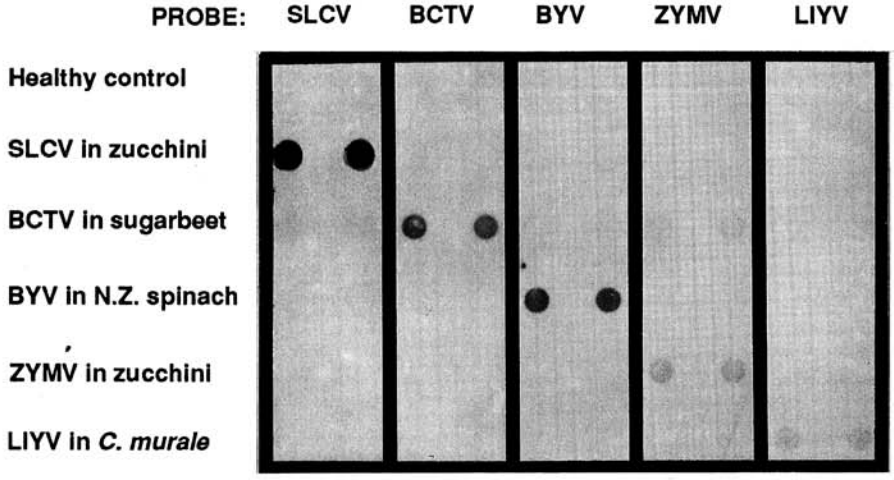

Fig. 1. Dot blot hybridization detection of plant viruses with digoxigenin-labeled probes detected colorimetrically. Squash leaf curl (SLCV) and beet curly top geminiviruses (BCTV), beet yellows closterovirus (BYV), zucchini yellow mosaic potyvirus (ZYMV), and lettuce infectious yellows virus (LIYV). All samples were processed with a leaf squeezer, homogenization buffer was added, and buffered homogenates were extracted with phenol/chloroform and denatured.

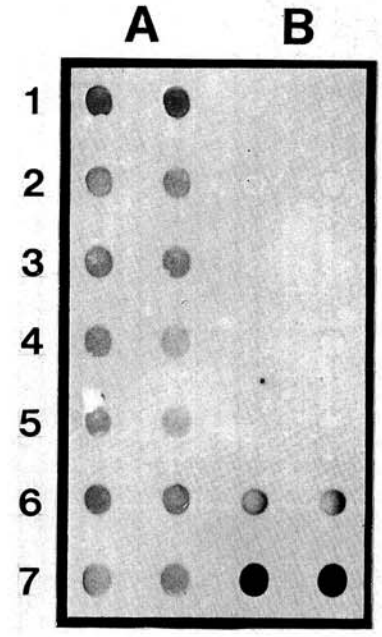

Fig. 2. Comparison of sample preparation procedures. Samples were applied to nitrocellulose in horizontal pairs of dots. Column A: tissue was homogenized with sample buffer, immediately denatured, and centrifuged; column B: tissue was homogenized, extracted with phenol/chloroform, and denatured. Rows of dots were from the following leaf-tissue sources: 1, healthy bean; 2, healthy cucumber; 3, healthy tomato; 4, zucchini yellow mosaic potyvirus-infected zucchini; 5, healthy tomato; 6, squash leaf curl geminivirus (SLCV)-infected bean; and 7, SLCV-infected zucchini. The membrane was hybridized to a digoxigenin-labeled SLCV probe and visualized colorimetrically.

Colorimetric visualization of immuno-detected nucleic acids was done with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate reagents, as recommended by Boehringer, for 30 min to 16 h. Color development was stopped by washing the membranes briefly in distilled water. Membranes were photographed wet.

Chemiluminescent detection was done by placing the equilibrated membrane between two sheets of clear acetate while still wet and slowly adding 0.5 ml/100 cm^3 of Lumi-Phos 530 (Lumigen, Inc., Detroit). After carefully distributing the Lumi-Phos over the membrane, the membrane was incubated overnight. Results were visualized and recorded by exposure of the membrane to film (Kodak X-Omat AR) at room temperature for 10 min.

RESULTS AND DISCUSSION

Dig-labeled probes detected a variety of viruses in plant tissues by dot blot hybridization (Fig. 1). The dig-labeled probes combined with colorimetric visualization were capable of detecting different types of viruses with a high degree of specificity, although improvement in the intensity of the signal or colorimetric reaction would be desirable for some virus/plant-tissue combinations. Little cross-reactivity resulting from the use of the digoxigenin-colorimetric system was apparent. However, certain plants, such as sugar beets, routinely gave high background with this technique, emphasizing the need for healthy plant controls.

The main obstacle to the use of the digoxigenin system for field samples is the binding of substances in crude plant-tissue homogenates (in addition to nucleic acids) to the hybridization membrane. Pigmentation on the blots confuses the interpretation of color development. Moreover, bound substances can either completely block or interfere with colorimetric detection, causing false reactions.

The importance of removing competing or interfering substances from field-sample extracts prior to use of the digoxigenin system for virus detection is illustrated by Figure 2. Although all the crude homogenates appear to exhibit the presence of SLCV, this effect is due to pigment in the samples prior to hybridization of the membrane. This color remained unchanged in response to hybridization and visualization; actual colorimetric detection of the virus was essentially blocked, although a "false signal" might be misinterpreted due to the presence of the plant pigment. When interfering substances were removed by phenol extraction, only the samples actually containing SLCV were clearly detected.

For the digoxigenin system to be a useful alternative for detecting plant viruses, the sensitivity needs to approach that of radioisotope labeling. As illustrated in Figure 3, the digoxigenin system can be employed to detect viruses in field samples with one-half the sensitivity of a ^{32}P-labeled probe. However, since use of the digoxigenin system allowed clear colorimetric detection of a 16-fold dilution of an average extract (derived from 1 to 2 g of tissue), the difference in sensitivity is probably not important for this application.

The use of Lumi-Phos for visualization may increase the clarity of the signal. However, as indicated by the manufacturer and shown in Figure 3C and D, considerable loss of signal occurs when Lumi-Phos detection is attempted with nitrocellulose membranes compared to nylon membranes. In contrast, the use of nitrocellulose membranes results in greatly reduced background with colorimetric visualization. Another difficulty with using Lumi-Phos for detection is that the visualization is completely blocked by residual phenol or homogenization buffer in plant extracts. This required that such samples be concentrated by ethanol precipitation after extraction with phenol/chloroform and then be reconstituted in TE buffer, pH 7.6 (11) to be detected in this manner. This extra purification step is not necessary for colorimetric visualization.

Satisfactory detection of tomato mottle geminivirus (data not shown) and SLCV was obtained when plant tissue (tomato, bean, and squash) was extracted with a homogenization solution (13) of 0.5 M sodium sulfite and 1% SDS (Fig. 4). However, difficulty was encountered when other kinds of viruses were extracted in this manner. As illustrated by Figure 4, the composition of the homogenization solution can have a significant effect on whether a particular virus can be extracted and subsequently detected by the digoxigenin system. The sodium sulfite solution and the Tris buffer functioned equally well with or without the addition of 2-mercaptoethanol for either of the two geminiviruses, even though SLCV was extracted from zucchini and BCTV was derived from sugar beet. However, neither variation of the sodium sulfite solution was effective for detection of BYV in New Zealand spinach or LIYV in *C. murale*. Furthermore, while BYV could be detected in New Zealand spinach processed in either of the Tris-based buffers, addition of 2-mercaptoethanol was

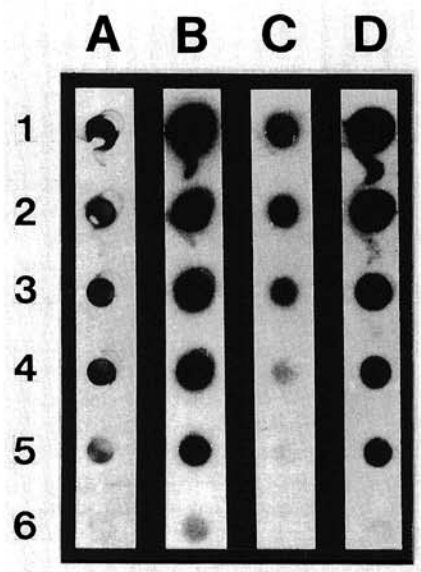

Fig. 3. Comparison of the sensitivity of radioactive and digoxigenin detection systems. All membranes were blotted with an identical series of dilutions from squash leaf curl geminivirus (SLCV)-infected zucchini. Row 1, undiluted material; row 2, 1:2 dilution; row 3, 1:4 dilution; row 4, 1:8 dilution; row 5, 1:16 dilution; and row 6, 1:32 dilution. Membranes A–C were nitrocellulose, and membrane D was nylon. Membranes A, C, and D were hybridized to a digoxigenin-labeled SLCV probe; membrane B was hybridized to a ^{32}P-dCTP-labeled probe. Membrane A, colorimetric visualization; membrane B, visualization by direct autoradiography; and membranes C and D, chemiluminescent visualization by autoradiography. Samples were treated as in Figure 1 and were then concentrated with ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA) prior to blotting.

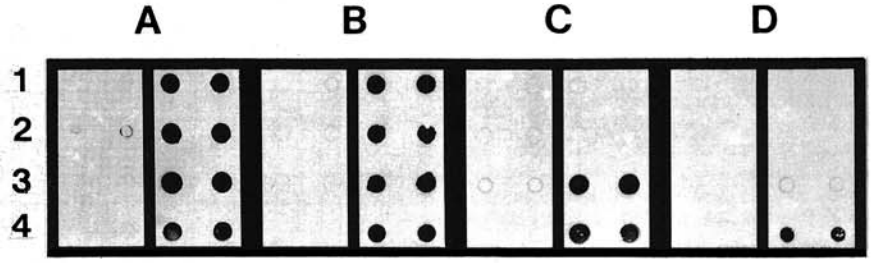

Fig. 4. Comparison of tissue extraction solutions. Row 1, tissue extracted in phenol/chloroform in solution containing 0.5 M sodium sulfite, 1% sodium dodecyl sulfate; row 2, tissue extracted as above with the addition of 4% β-mercaptoethanol; row 3, tissue extracted in Tris buffer; and row 4, tissue extracted in Tris buffer containing β-mercaptoethanol. Panel A, extracts from healthy and squash leaf curl geminivirus-infected zucchini; panel B, extracts from healthy and beet curly top geminivirus-infected sugar beet; panel C, extracts from healthy and beet yellows closterovirus-infected New Zealand spinach; and panel D, extracts of healthy and lettuce infectious yellows-infected *Chenopodium murale*. On each pair of nitrocellulose membranes (panel), paired dots of extract from healthy tissue were applied vertically on the left; infected material was blotted in duplicate on the right. Extracted samples were hybridized with respective probes and visualized colorimetrically.

clearly required for detection of LIYV in *C. murale*.

All of these viruses can be detected in blots derived from crude plant homogenates by hybridization using radioactively labeled probes. However, because phenol/chloroform extraction seems to be one effective approach for minimizing the presence of plant-derived substances that would otherwise interfere with the digoxigenin system, buffer composition can become an important consideration in optimizing the usefulness of this detection method for a given virus system.

Protease treatment of membranes blotted with unextracted plant homogenates was tested as an alternative to solvent extraction of plant samples for removal of substances that interfere with dig-labeled detection and visualization. Although the results varied with the different viruses tested, this approach was successful for some systems. LIYV-infected samples were more clearly detected colorimetrically when the blotted nitrocellulose membrane was treated with Proteinase K after baking and prior to hybridization compared to the nonprotease treated controls. However, the colorimetric signal intensity and background color control for Proteinase K-treated blots was not as satisfactory as for blots of phenol-extracted samples for any tested virus, and SLCV in particular was barely detectable using this method. Slightly better resolution of infected samples could be attained if the blots were baked than if the samples were UV-linked to the membrane, and if the Proteinase K treatment was done after baking rather than before (data not shown). Treating the blotted membranes with Pronase alone or in addition to proteinase, treating membranes twice with either Proteinase K, or conducting the enzymatic treatment at 50°C did not substantially improve resolution (data not shown).

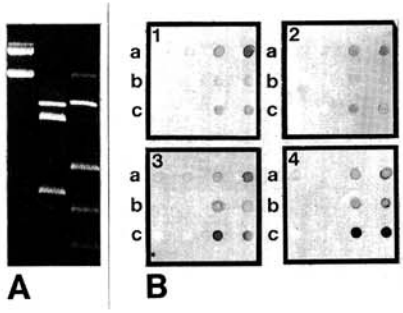

Fig. 5. Comparison of lettuce infectious yellows virus (LIYV) probe preparation methods. **(A)** Ethidium bromide-stained 1% agarose gel showing digestion of pL105. Undigested purified pL105 (left lane), *Mlu*I digest (middle lane), and *Xba*I digest (right lane). **(B)** Identical nitrocellulose membranes blotted with formaldehyde/SSC-heat denatured extracts of: row a, *Chenopodium murale*; row b, *C. quinoa*; and row c, *Nicotiana benthamiana*. Horizontal pairs of dots on the left of each membrane were from healthy tissue; pairs on the right were from LIYV-infected tissue. Membranes were hybridized with digoxigenin-labeled probes derived from the complete *Mlu*I digest (membrane 1), the complete *Xba*I digest (membrane 2), the small pL105 segment from the *Mlu*I digest (membrane 3), and the large pL105 segment (middle band on gel) from the *Mlu*I digest (membrane 4). All were visualized colorimetrically.

Use of nylon membranes in conjunction with Lumi-Phos visualization improved detection of dig-labeled probes hybridized to enzymatically treated blots of denatured plant homogenates compared to colorimetric methods. Some of the difference in effectiveness between the two visualization methods may be due to the ability of interfering substances remaining in such blots to chemically reduce the nitroblue tetrazolium but not to affect light emission (7).

Of the viruses tested with the digoxigenin system, LIYV was the most difficult to detect. However, we found that radioactively labeled probes generated from the same pL105 clone used for dig-labeling also poorly discriminated LIYV. In contrast, the dig-labeled probe for BYV was highly effective.

Some of the differences in performances of the clones as sources of probes could be due to differences in virus concentration in plants rather than the quality of the clone probes. The relatively small size of the BYV probe also may have improved its efficiency in nonradioactive detection systems in which steric hindrances could be a problem (10).

In addition, the pSport vector of the pL105 clone of LIYV might competitively interfere with dig-labeling if the plasmid segment is retained in the restriction digest after excision of the clone segment(s). This could occur, for example, if the plasmid segment happened to have a high affinity for the random primers or many possible substitution sites for the dig-dUTP.

Consequently, in an attempt to improve performance of pL105 as the source of a probe in the digoxigenin detection system, the clone was digested into multiple small pieces using restriction enzymes, or the complete excised clone segment was separated from the digestion mixture before dig-labeling. Merely labeling smaller viral clone segments produced by *Xba*I digestion gave no enhancement over probes produced from the complete *Mlu*I digest. However, separating the clone segments of the *Mlu*I digest from the pSport segment prior to dig-labeling did improve the performance of the resulting probes, particularly the one derived from the main pL105 segment (Fig. 5).

Radioactive probes derived from the same viral clones used to generate the dig-labeled probes in this study have been effective in detecting viral nucleic acids in blots of plant extracts without additional digestion of the clone or phenol extraction of the plant extracts. The additional treatments required for effective use of dig-labeled probes may be due in part to steric hindrance imposed on the probe by the presence of the digoxigenin moiety (10).

The results shown in Figure 5 suggest that increasing the degree of separation of the clone to be labeled from its associated vector plasmid improves the performance of dig-labeled probes. Complete excision of viral clones from their vectors resulted in more effective probes than merely linearizing the clone-containing plasmid, even when the detached vector segment was present during labeling and hybridization. Additional improvement in signal intensity of the LIYV probe was noted when the pL105 clone was removed from the vector segment prior to labeling (Fig. 5). Increasing the separation of the clone segment from its vector may simply increase the direct accessibility of the resultant probe to its target sequences. However, the performance of a BCTV probe derived from a linearized clone-containing vector was increased to the same level as a probe produced from a completely excised clone by adjusting the linearizing cut such that the clone portion was exposed at one end of the plasmid rather than between portions of the vector (data not shown).

Our data suggest that the most effective nonradioactive probe for plant virus detection would be a clone segment that was excised from its purified vector plasmid, while further separation of the clone segment could be helpful, depending on the performance of the probe. If the specificity of the probe is maintained, there may be an advantage in reducing the size of the labeled probe for nonradioactive detection methods.

Although preparation of viral probes requires a well-equipped laboratory, we have found that laboratories with access to an available probe can readily adapt this detection method for use with minimal equipment. Although far from the recommended approach, we have detected geminivirus colorimetrically from field samples, using a dig-labeled probe provided by our laboratory in a remote field station, without using a specialized blotting apparatus, a vacuum for blotting or baking, or an incubator with temperature control and using periodic agitation by hand. We feel that this procedure has potential merit for routine testing in small diagnostic laboratories.

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