Digoxigenin-Labeled cRNA Probes for the Detection of Two Potyviruses Infecting Peanut (*Arachis hypogaea*)

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ABSTRACT

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Nonradioactive dot blot hybridization and chemiluminescent detection were used for the diagnosis of peanut mottle (PeMoV) and peanut stripe (PStV) potyviruses. Digoxigenin-labeled cRNA probes corresponding to the 3′ terminal 1,400 (PeMoV) and 1,700 (PStV) nucleotides were transcribed from recombinant cDNA clones. Both viruses were detected in the picogram range in purified preparations and in infected peanut leaf extracts. No cross-hybridization between PStV and PeMoV was observed with either probe under conditions of high stringency. PStV cRNA probes of the complete coat protein gene and 3′ untranslated region cross-hybridized with bean common mosaic virus, confirming the close relationship of these two viruses. However, a 300-nucleotide probe corresponding to the variable amino terminus of the coat protein was specific for PStV.

Peanut stripe virus (PStV) and peanut mottle virus (PeMoV) are two economically important viruses infecting peanut. PStV is widespread in China and Southeast Asia, and PeMoV occurs worldwide (4,8,9,26). Both viruses are members of the family Potyviridae, are seed transmitted in peanut, and are transmitted by aphids in a nonpersistent manner (2,4,9). PStV is readily transmitted through peanut seed at a rate of up to 20% (6,10,26) and has apparently been introduced into the United States, India, and some African countries through peanut germ plasm exchange (8). Transmission rates in peanut seed of 0–5% have been reported for PeMoV (2).

At present, control of the two virus diseases depends on integrated measures, including the use of virus-free peanut seeds and the prevention of further virus spread through seed movement by adoption of quarantine measures (10). The availability of sensitive detection systems for PStV and PeMoV is therefore essential. Different formats of enzyme-linked immunosorbent assay (ELISA) have proved to be a reasonably sensitive and reliable means for detection of both viruses (2,10,15,19,26), particularly when monoclonal antibodies were used (6,7, 20). However, a degree of serological cross-reactivity with some other potyviruses has been reported (7,25). Furthermore, only relatively few seeds (about five to 30) can be combined per sample without loss of sensitivity (2,6,10). Nucleic acid hybridization is a powerful technique for detection of specific complementary nucleic acid sequences (16) and is being increasingly used for potyvirus detection (3,13,22).

The use of radioactively labeled complementary (cDNA) probes for the detection of PeMoV and PStV in peanut seed has improved the sensitivity eight- to 10-fold over ELISA (3). ^{32}P-labeled cDNA probes can reliably detect one part infected seed when mixed with 99 parts of healthy seeds (3). However, radioactively labeled probes have the combined disadvantages of short shelf life and health hazards during use and disposal. Various nonisotopic labels like biotin (11) or digoxigenin (DIG) (14,24) are available as alternatives to radioisotopes. The DIG labeling procedure provides a sensitivity comparable to radiolabeling (14,24) with a shorter time for development of the signal. Chemiluminescent detection offers increased sensitivity over colorimetric substrates (12,14) and eliminates interference by stains from plant tissue extracts. Complementary RNA probes have the advantage of being single-stranded and form more stable hybrids than DNA probes (5). Here we present the application of DIG-labeled cRNA probes generated by in vitro transcription of virus-specific cDNA clones for the detection of PStV and PeMoV in peanut leaf tissue.

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MATERIALS AND METHODS

Virus isolates. PStV was isolated from an infected seed imported from Indonesia and propagated in peanut (*Arachis hypogaea* L.) under quarantine conditions. An Australian peanut isolate of PeMoV which had been passaged through single local lesions in bean (*Phaseolus vulgaris* L.) cv. Kerman was propagated in bean cv. Bountiful. Both viruses were purified as described by Demski (9). Isolates of bean common mosaic virus (BCMV) from bean, soybean mosaic virus (SMV) from soybean (*Glycine max* (L.) Merr.), passionfruit woodiness virus (PWV) from corky passion vine (*Passiflora suberosa* L.), blackeye cowpea mosaic virus (BlCMV) from cowpea (*Vigna unguiculata* (L.) Walp.), and potato virus Y (PVY) from tobacco (*Nicotiana tabacum* L.) cv. Xanthi nc from the Queensland Department of Primary Industries (QDPI) collection of viruses were propagated on their respective hosts.

Sample preparation. Purified virus preparations of known concentration were diluted in denaturation buffer (12 × SSC [1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 6% formaldehyde). Aqueous extracts of infected and noninfected plant tissue were prepared by grinding in 50 mM citrate buffer, pH 8.3 (1:4 w/v). Extracts were subsequently diluted fivefold in denaturation buffer, as described by Varveri et al (22). Two other methods of sample preparation were evaluated: 1) 100 mM Tris, pH 7.6, containing 1% sodium dodecyl sulfate (SDS) as extraction buffer, followed by denaturation in 7% formaldehyde at 65 C for 15 min (9) and 2) extraction in 0.2 M potassium phosphate, pH 7.4, containing 1% SDS and 0.1% sodium sulfite, and subsequent phenol/chloroform (1:1) extraction. One microliter of each sample was applied to a nylon membrane (Hybond N, Amersham Corp., Sydney, Australia), which had been rinsed first with diethyl pyrocarbonate-treated water, then with 20 × SSC, and air-dried. Nucleic acids were fixed to the membrane by UV cross-linking with an energy of 50 mJ.

Nonradioactive dot blot hybridization assay. Four recombinant plasmids were used for cRNA probe synthesis. The probes pPeMoV and pPStV were complementary to the 3′ terminal 1,400 nucleotides (nt) and 1,700 nt, respectively, of either virus (21). These probes each contained sequences corresponding to the 3′ end of the NIb gene, the complete coat protein (CP) gene, and the 3′ untranslated region (Fig. 1). The cRNA probes pPStV-N and pPStV-C contained sequences corresponding to the amino and carboxy termini, respectively, of the CP (Fig. 1). DIG-labeled RNA complementary to the viral genome were transcribed from linearized pBluescript cDNA clones containing 3′ terminal sequences of PStV and PeMoV, using T_3 or T_7 RNA polymerase, respectively, and DIG-UTP, according to the manufacturer's directions (Boehringer Mannheim Biochemicals, Castle Hill, NSW, Australia). Labeling efficiency of cRNA probes was confirmed by dot blot detection assay, and probe concentration was determined spectrophotometrically. Samples were applied to nylon membrane as described above. Hybridization was done at 68 C in 5 × SSC, 50% formamide, 0.02% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent, and DIG-labeled cRNA at 0.5 μl/ml. The membranes were washed twice for 5 min at room temperature (RT) in 2 × SSC, 0.1% SDS, and twice for 15 min at 68 C in 0.2 × SSC, 0.1% SDS. Nonspecific binding sites were blocked for 1 hr at RT in blocking solution (10% blocking reagent, 0.1 M maleic acid, and 0.15 M NaCl, pH 7.5) diluted fivefold in 0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl. The membranes were then incubated for 30 min in anti-DIG IgG APase conjugate (1:10,000 dilution), washed, and equilibrated in the appropriate buffers. Following incubation with Lumigen PPD, the damp membranes were sealed between sheets of transparent plastic film and exposed to X-ray film for 2–4 hr.

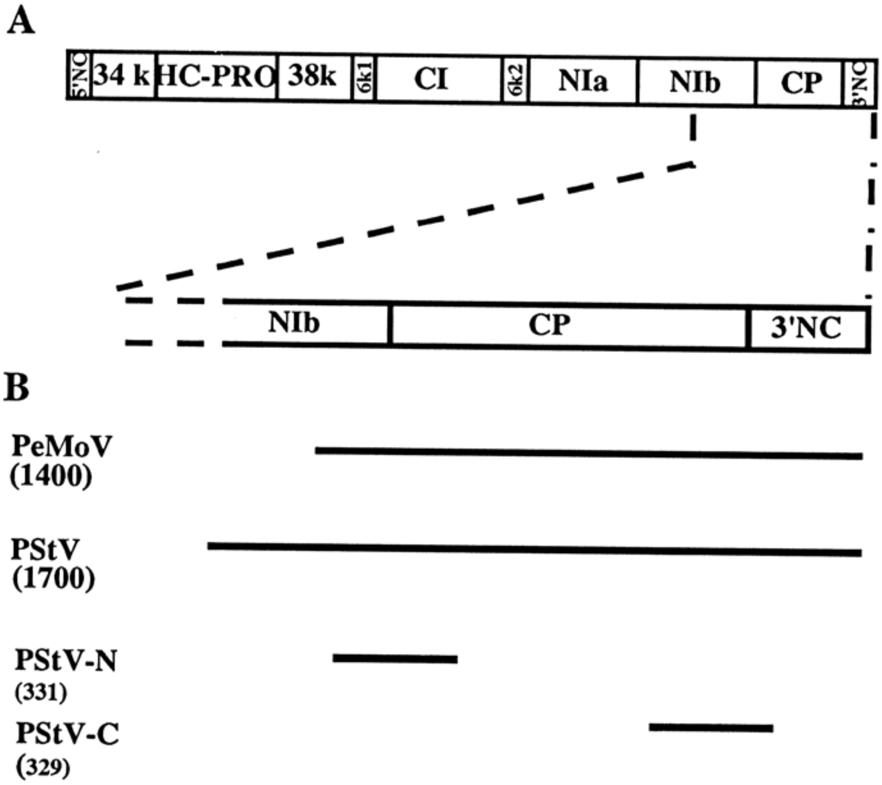

Fig. 1. Location of cRNA probes relative to the potyvirus genome. **(A)** General organization of the potyvirus genome. **(B)** Location and length of cRNA probes to peanut mottle (PeMoV) and peanut stripe (PStV) viruses. Length of the probes in nucleotides (nt) is given in parentheses.

RESULTS

Probe synthesis and sample preparation. The three DIG-labeled PStV cRNA probes and one PeMoV cRNA probe were synthesized by in vitro transcription. From 14 to 40 μg of labeled cRNA probe were obtained from 1 μg of plasmid DNA. Labeling efficiency of all probes was usually as high as the control-labeled RNA probe provided by the manufacturer. Our leaf sample preparation method was quick and easy and gave consistently good results and the highest sensitivity of the three extraction methods used (data not shown).

Sensitivity and specificity of PStV detection. All three PStV cRNA probes hybridized to purified homologous virus and virus-infected leaf extracts. The 1,700-nt PStV cRNA probe detected as little as 250 pg of purified PStV (Fig. 2A) or 1:2,560 dilution of PStV-infected peanut leaf extract (Fig. 3A). This probe also hybridized to bean leaf extracts infected with the closely related BCMV, but not above a 1:20 dilution (Fig. 3A). However, the 1,700-nt PStV cRNA probe did not hybridize with other members of the BCMV subgroup at this dilution. Furthermore, it did not hybridize with 160 ng of purified PeMoV (Fig. 2A), or with 1:20 diluted PeMoV-infected plant extracts, or with RNA extracted from healthy plant tissues (Fig. 3A).

The specificity and sensitivity of the 1,700-nt cRNA probe which contained the complete CP gene and the 3′ untranslated sequence of PStV was compared with the shorter 300-nt probes which corresponded to the N- or C-terminal region of the CP (Fig. 4). Probes PStV and PStV-C exhibited a similar degree of sensitivity by detecting PStV in 1:2,500 diluted peanut leaf extract, whereas the detection limit using PStV-N was fivefold less (Fig. 4, lanes 1). Probes PStV and PStV-C cross-hybridized with BCMV at a dilution of 1:20 (Fig. 4A and C, lanes 2), whereas PStV-N appeared to be specific for PStV (Fig. 4B, lane 2). When blots were exposed to film for 2–4 hr, none of the cRNA probes hybridized with the other potyviruses tested or with extracts from uninfected leaves. However, a weak reaction of only the 1,700-nt PStV probe with PWV was detected when blots were exposed to X-ray film for more than 16 hr. No signal was detected using the longer exposure with any of the other viruses or uninfected extracts (data not shown).

Sensitivity and specificity of PeMoV detection. The PeMoV cRNA probe hybridized to as little as 10 pg of purified

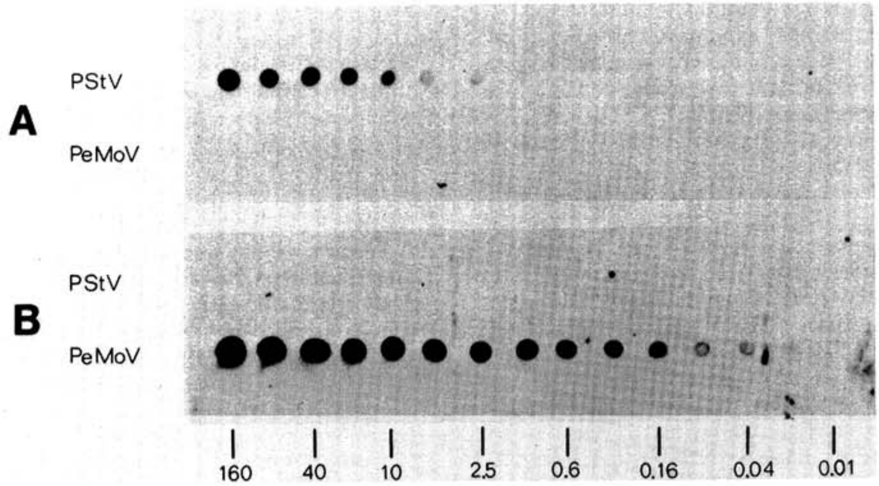

Fig. 2. Detection of peanut potyviruses in purified preparations by dot blot hybridization. Twofold dilution series of peanut stripe (PStV) and peanut mottle (PeMoV) viruses were hybridized with cRNA probes **(A)** PStV or **(B)** PeMoV. Virus concentrations (ng) given below were estimated spectrophotometrically.

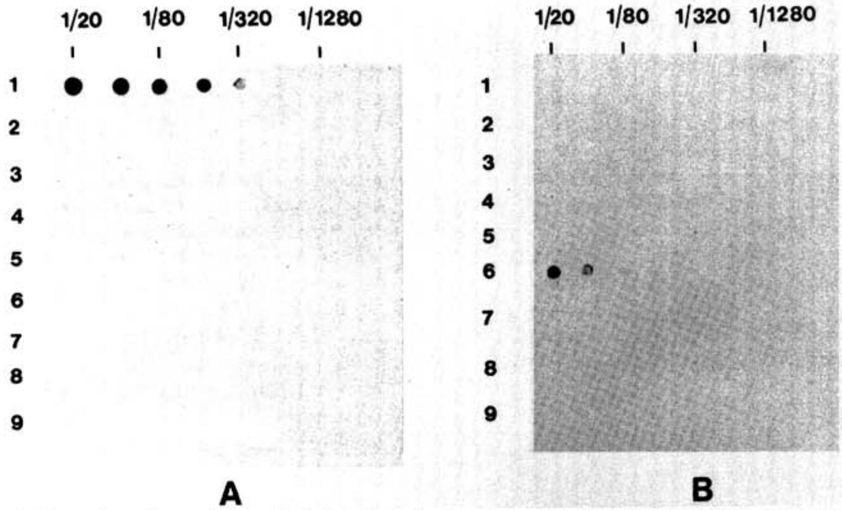

Fig. 3. Detection of potyviruses in infected leaf extracts by dot blot hybridization. Twofold dilution series from 1/20 to 1/2,560 were hybridized with cRNA probes to either **(A)** peanut stripe (PStV) or **(B)** peanut mottle (PeMoV) viruses. Samples were (1) PStV-infected peanut, (2) bean common mosaic virus–infected bean, (3) blackeye cowpea mosaic virus–infected cowpea, (4) soybean mosaic virus–infected soybean, (5) passionfruit woodiness virus–infected bean, (6) PeMoV-infected peanut, (7) potato virus Y–infected tobacco, (8) uninfected peanut, and (9) uninfected bean.

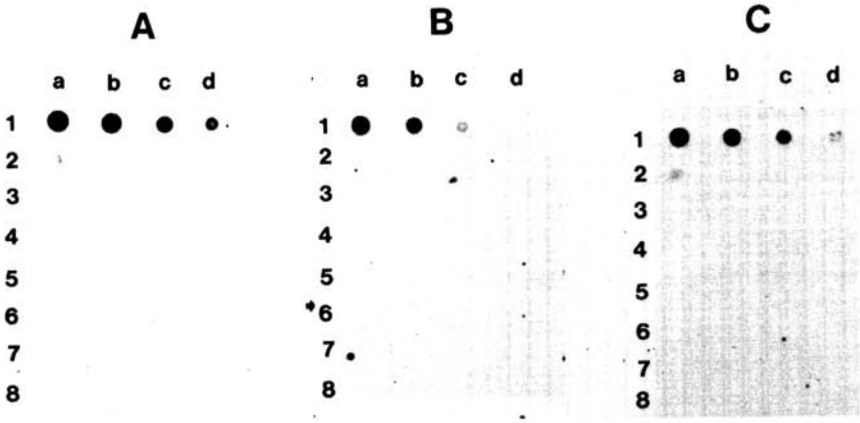

Fig. 4. Comparison of the specificity of three peanut stripe virus (PStV) cRNA probes in dot blot hybridization. Fivefold dilution series of virus-infected and uninfected leaf extracts were hybridized with cRNA probes **(A)** PStV, **(B)** PStV-N, and **(C)** PStV-C. Dilutions tested were (a) 1/20, (b) 1/100, (c) 1/500, and (d) 1/2,500. Samples were (1) PStV-infected peanut, (2) bean common mosaic virus–infected bean, (3) blackeye cowpea mosaic virus–infected cowpea, (4) soybean mosaic virus–infected soybean, (5) passionfruit woodiness virus–infected bean, (6) peanut mottle virus–infected peanut, (7) uninfected peanut, and (8) uninfected bean.

homologous virus (Fig. 2B) and to extracts from PeMoV-infected bean (cv. Bountiful) up to a dilution of 1:160 (Fig. 3B). No hybridization with purified PStV was detected even at a concentration of 160 ng (Fig. 2B). The PeMoV cRNA probe failed to hybridize with RNA from uninfected plant tissues or extracts of leaves infected with other potyviruses, namely PStV, BCMV, BlCMV, PWV, SMV, and PVY (Fig. 3B).

DISCUSSION

Our results show that in vitro transcribed DIG-labeled PStV and PeMoV cRNA probes are sensitive and specific tools for the detection of potyviruses infecting peanut. The 1,700-nt PStV cRNA probe can detect as little as 250 pg of purified PStV (equal to about 10 pg of PStV RNA) or 1:2,500-diluted extracts of PStV-infected peanut leaves. Therefore, the chemiluminescent detection assay described here provides a sensitivity similar to radioactive assays (3). PStV was detected in 1:62,500 diluted seed extract using a ^{32}P-labeled PStV cDNA probe (3). Similar results were reported by Fouly et al (12), who compared the sensitivity of an in vitro transcribed biotin-labeled barley yellow dwarf virus RNA probe and a ^{32}P-labeled cDNA probe using dot blot hybridization. Eweida et al (11) reported that a biotin-labeled potato virus X probe could detect as little as 1 pg of purified PVX. Both DIG- and biotin-labeled RNA or DNA probes used chemiluminescence for detection of nucleic acids. These methods provide safe and sensitive assays and may allow the wider use of nucleic acid probes in laboratories which do not have facilities for handling radioisotopes.

Our data indicate that DIG-labeled cRNA probes which cover the entire CP gene and the 3′ untranslated region are highly specific under our conditions of stringency. We did not detect nonspecific reactions with healthy plant extracts. PStV cRNA probes did not cross-hybridize with PeMoV RNA, and vice versa, even though the two viruses share 64.4% nucleotide sequence homology in the CP gene region (21). PeMoV cRNA probe did not hybridize with other potyviruses, namely PWV, BCMV, SMV, BlCMV, and PVY. The 1,700-nt PStV cRNA probe detected BCMV which is serologically related to PStV but did not detect BlCMV which is closely related (17,18). Use of the shorter 300-nt probe, which corresponded to the hypervariable amino terminus of the CP, provided a tool specific for the detection of PStV. Differentiation of potyviruses and their strains has previously been achieved using probes corresponding to the 3′ untranslated region (13). This region is part of the 1,700- and 1,400-nt probes used for the detection of PStV and PeMoV, respectively. PStV and some strains of BCMV were recently identified as strains of the same virus on the basis of high homology of their coat protein peptide profiles (17,18), host range, and serology (23). A proposal is under consideration by the International Committee for the Taxonomy of Viruses that PStV be reclassified as peanut strain of BCMV (P. Berger, *personal communication*).

We adopted a sample preparation method described by Varveri et al (22) for dot blot hybridization. In comparison with other sample preparation methods, this method is simple and consistently gave good results with leaf tissue. Maule et al (16) and Baulcombe et al (1) showed that it is not necessary to pretreat virus preparations to disrupt the virus capsids in order for the nucleic acid to bind to the nitrocellulose. In preliminary tests, we found that the direct use of PeMoV- and PStV-infected leaf tissue extracted and diluted in citrate buffer without subsequent denaturation with formaldehyde gave identical results. This further simplifies sample preparation. However, seed extracts did not yield reliable results and appeared to inhibit hybridization or chemiluminescent detection. Improved extraction methods for peanut seeds which involve precipitation of viral RNA are currently under investigation.

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