Sensitive Broad-Spectrum Detection of Indian Peanut Clump Virus by Nonradioactive Nucleic Acid Probes

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ABSTRACT

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Cloned cDNA fragments representing different regions of the genome of the H serotype of Indian peanut clump furovirus (IPCV) were used as hybridization probes. Dot-blot assays using digoxigenin-labeled probes readily detected IPCV in extracts from infected plants with a sensitivity approaching that of using ^{32}P-labeled probes. The specificity of the probes depended on the part of the IPCV genome represented in the cDNA. Two probes representing the coat protein gene and adjacent gene in RNA-2 readily detected infection by H serotype IPCV, but reacted poorly with samples of plants infected with other serotypes. A probe corresponding to sequence near the 5′-end of RNA-1 reacted with samples of all IPCV serotypes, but not with those of peanut clump virus (PCV) from West Africa. A probe corresponding to the 3′-terminal 742 nucleotides of RNA-1 readily detected RNA of any IPCV serotype and of PCV. Assays using this probe, labeled with a nonradioactive reporter-digoxigenin, could detect IPCV in samples of seed tissue, as well as in tissue of several weed species and crop species such as wheat. The probe did not react with samples from plants infected by other furoviruses.

Peanut clump disease is caused by infection with Indian peanut clump furovirus (IPCV) in India (16) or peanut clump virus (PCV) in Africa (19). IPCV and PCV belong to the genus *Furovirus* (11). They possess bipartite, positive-sense RNA genomes (10,17) and are transmitted by the fungus *Polymyxa graminis* Ledingham (13,15,20). The disease causes severe yield losses in groundnut (15) and affects other legume crops, as well as cereal crops. The viruses are seed-transmitted (7,14). There is little serological relationship between IPCV and PCV, and IPCV occurs as three serotypes that are distinct even when polyclonal antibodies are used for the tests (12). The amino acid sequences of the coat proteins of an isolate of PCV and an isolate of the H serotype of IPCV were 61% identical (22).

The lack of serological cross-reaction among the three IPCV serotypes complicates disease surveys and diagnostic work such as in quarantine tests. For such tests, an ideal detection reagent would have a sufficiently broad specificity to detect all serotypes and would also be as widely applicable as possible by being based on nonradioactive assay methods. We describe here the development of a broad-specificity nucleic acid probe, labeled with the nonradioactive reporter molecule digoxigenin, which can detect all the currently known serotypes of IPCV, as well as an isolate of PCV, in extracts of plant tissues including peanut seed. This is the first report of development of such a broad-specificity diagnostic assay for use in the detection of IPCV or PCV.

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MATERIALS AND METHODS

Virus isolates and field samples. The isolates of IPCV from Hyderabad, Andhra Pradesh (IPCV-H); Talod, Gujarat (IPCV-T); and Ludhiana, Punjab (IPCV-L); and PCV from West Africa were those described by Reddy et al. (17) and were maintained in *Phaseolus vulgaris* L. The identity of each isolate was verified by enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (12). Virion purification and extraction of RNA were as described elsewhere (17). Samples of other furoviruses and healthy control tissue were obtained from the Agricultural Experimental Station, Orléans, France (soilborne wheat mosaic virus); Université Catholique de Louvain, Louvain-la-Neuve, Belgium (beet necrotic yellow vein virus); and the Scottish Crop Research Institute, Dundee, United Kingdom (potato mop-top virus).

Plants. Peanut plants and the weed species *Eragrostis uniloides* (Retz.) Nees ex Steud., *Digitaria ciliaris* (Retz.) Koeler, *Cyperus diffusus* Vahl, *Cyperus rotundus* L., and *Cynodon dactylon* (L.) Pers. were collected from different fields containing IPCV-infected groundnut plants located in the Indian states of Andhra Pradesh (Hyderabad, Pallepalem, and Bapatla) and Rajastan (Durgapura, Boraj, and Dausa).

Sampling of seed tissue. Small portions of the cotyledons were excised from each seed so as to leave the embryonic axle untouched, and weighed amounts were processed either for ELISA or for the extraction of nucleic acid. The seeds were then allowed to germinate. Samples were taken from germinated seedlings, and extracts were assayed for the presence of IPCV coat protein by ELISA.

ELISA. Samples were assayed by the double-antibody sandwich method (DAS-ELISA) using penicillinase-conjugated antibodies as previously described (18). High values in this assay indicate a lack of serological reaction (18).

Hybridization probes. Cloned cDNA of IPCV RNAs obtained in previous studies (21,22; J. S. Miller, *unpublished data*) was used as a source of probes. Figure 1 illustrates the location of the probe sequences in the genome of IPCV. Probe 1 corresponded to the sequence from nucleotide position 44 to 1,130 of RNA-2, probe 2 corresponded to the sequence from position 1,310 to 2,098 of RNA-2, probe 3 corresponded to the sequence from position 655 to 1,270 of RNA-1, and probe 4 corresponded to the sequence from position 5,099 to 5,841 of RNA-1. DNA fragments were labeled either with ^{32}P-dCTP or with dUTP labeled with digoxigenin (DIG) (DIG DNA labeling and detection kit, Boehringer GmbH, Mannheim, Germany) by using the random primer method (5).

Preparation of plant samples for hybridization assay. Leaves were homogenized in a sterile mortar with 10 volumes of 50 mM sodium citrate, pH 8.3, as described by Mas et al. (9) or with STE buffer (0.1 M NaCl, 10 mM Tris [pH 8.0], and 1 mM EDTA [pH 8.0]) centrifuged at 10,000 × *g* for 10 min and serially diluted with the same buffer. Nucleic acids were extracted from leaves, roots, and seeds by grinding samples in extraction buffer containing 1 M Tris (pH 8.0), 200 mM LiCl, 2% sodium dodecyl sulfate (SDS), and 20 mM EDTA. The mixture was subsequently extracted once with Tris buffer-saturated phenol, and the nucleic acids in the aqueous phase were precipitated by the addition of 2.5 volumes of ethanol and $^{1}/_{10}$ volume of 3 M sodium acetate. After 2 h at –20°C, the mixture was centrifuged at 10,000 × *g* for 15 min, washed once with 70% ethanol, dried under vacuum, and resuspended in water. The concentration of nucleic acid was estimated spectrophotometrically at 260 nm.

Dot blotting. Samples (50 µl) containing either viral RNA (1 µg to 10 pg), different dilutions (10- to 1,000-fold) of leaf extract (1 g/ml), or different amounts of total nucleic acid (2 to 0.5 µg) extracted from leaves were applied to nylon membrane (Hybond N; Amersham, Buchinghamshire, United Kingdom) using a dot-blot apparatus (Bio-Rad Laboratories, Richmond, CA). The nucleic acids were bound to the membrane by exposing both sides of the membrane to UV light (0.12 J) in a UV crosslinker (Stratagene Inc., La Jolla, CA).

Hybridization conditions. For use with a radioactive probe, prehybridization was done for 2 to 3 h at 65°C in a hybridization oven (Hybaid, Teddington, United Kingdom) in 5× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) containing 0.02% SDS, Denhardt's reagent (0.04% each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidone), and 100 µg/ml of denatured calf-thymus DNA. After overnight hybridization with the probe, the membrane was washed twice (15 min each) with 2× SSC containing 0.1% SDS and twice (15 min each) with 0.5× SSC containing 0.1% SDS, dried, and exposed to the X-ray film (Indu, Hindustan Films Ltd., Mumbai, India) at –70°C with intensifying screens. For DIG-labeled probes, prehybridization was for at least 1 h at 68°C in a hybridization buffer containing 5× SSC, 0.1% N-lauryl sarcosine (wt/vol), 0.02% SDS (wt/vol), and 1% (wt/vol) blocking reagent (Boehringer GmbH). The probe was denatured by boiling for 10 min and added at a concentration of 300 ng/ml of fresh hybridization buffer. Hybridization was at 68°C overnight. When using the commercially available DIG Easy-Hyb solution (Boehringer GmbH), the hybridizations were at 50°C. The filters were washed twice (5 min each) in 2× SSC and in 0.1% SDS at room temperature, and then twice (15 min each) in 0.1× SSC and in 0.1% SDS at 68°C.

The DIG-labeled hybrids on the membrane were detected using anti-DIG Fab fragments conjugated to alkaline phosphatase (Boehringer GmbH). After hybridization, the DIG was detected either by reaction with 5-bromo,4-chloro,3-indolyl phosphate and nitrobluetetrazolium, which produced a purple precipitate, or in a chemiluminescent reaction with 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxatane disodium salt (Tropix Inc., Bedford, MA) as described by the manufacturer.

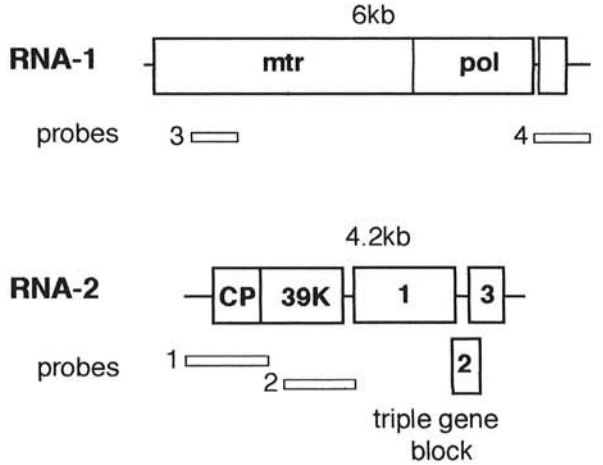

Fig. 1. A diagram of the genome of Indian peanut clump virus showing the locations of the probes (1 to 4). RNA is represented as a solid line and the boxes represent the putative protein products of translation of the RNAs. CP = coat protein; mtr = putative methyl transferase domain; and pol = putative polymerase domain.

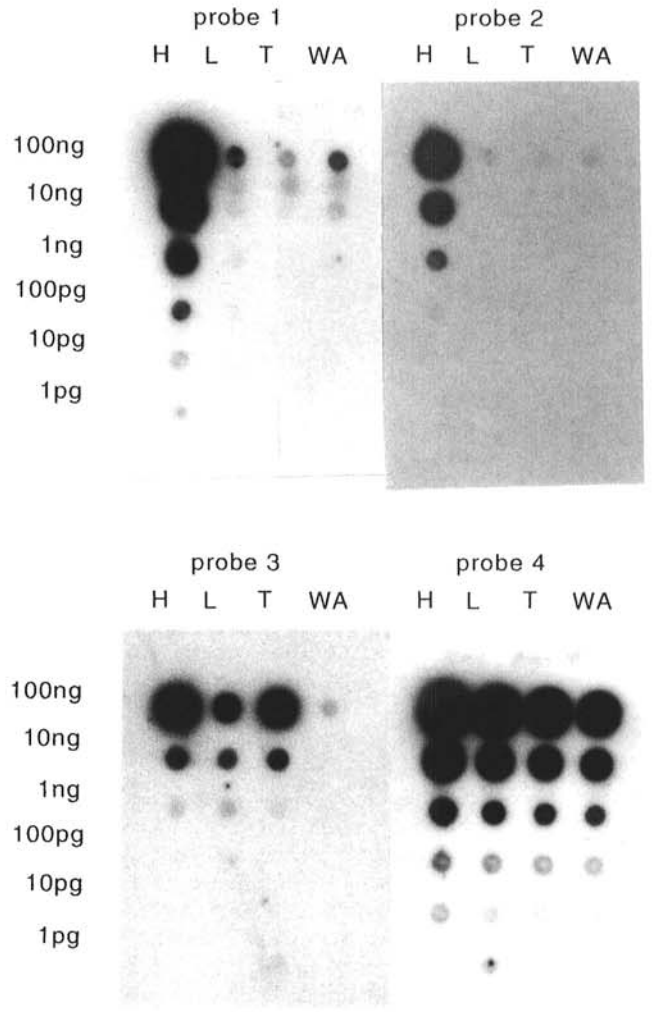

Fig. 2. Dot-blot hybridization assays of Indian peanut clump virus (IPCV) and peanut clump virus (PCV) RNA using probes 1 to 4. Known quantities (indicated on the left) of RNAs from purified preparations of IPCV-H (from Hyderabad [H]), IPCV-L (from Ludhiana [L]), IPCV-T (from Talod [T]), and PCV (WA) were hybridized to ^{32}P-labeled probes.

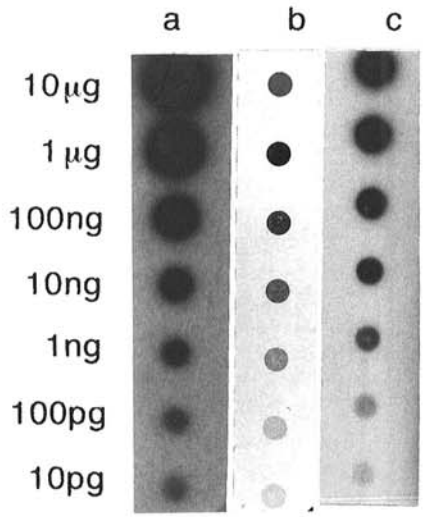

Fig. 3. Comparison of detection methods. Fifty-microliter samples containing known amounts of Indian peanut clump virus from Hyderabad RNA (indicated on the left) were spotted on the membrane and hybridized to ^{32}P-labeled probe and detected by autoradiography (lane a), digoxigenin-labeled probe using chromogenic (lane b), or chemiluminescent substrates (lane c).

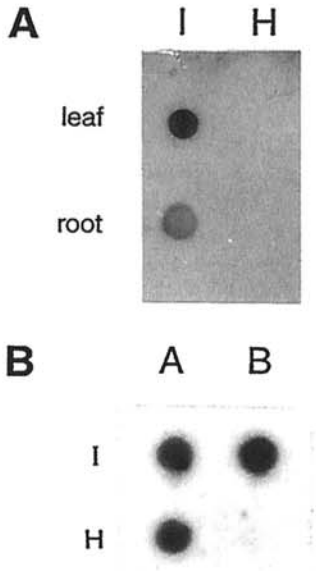

Fig. 4. Detection of Indian peanut clump virus (IPCV) infection. Samples of 50 µl of total RNA from **A,** leaves and roots of *Eragrostis uniloides* or **B,** leaves of wheat plants. *E. uniloides* was sampled from soil adjacent to IPCV-infected peanuts (lane I) or from soil not close to infected plants (lane H). Wheat plants were sampled from wheat seedlings growing where IPCV-infected peanuts had been grown previously.

RESULTS

Determination of specificity of the probes. To test the specificity of the cloned cDNA probes, cDNA fragments representing different parts of the IPCV genome (Fig. 1) were excised from the clones, labeled with ^{32}P-dCTP, and used as probes in dot-blot hybridization assays. Of the four probes tested, probes 1 and 2 reacted strongly with the homologous RNA and weakly with RNAs of other isolates at the highest concentration used (Fig. 2). Probe 3 reacted strongly with the homologous RNA, as well as with RNA of IPCV-T serotype, less strongly with IPCV-L, and only faintly with PCV. Probe 4 reacted strongly with RNA of all serotypes tested, the limit of sensitivity being about 10 pg with the heterologous RNA and about 1 pg with the homologous RNA.

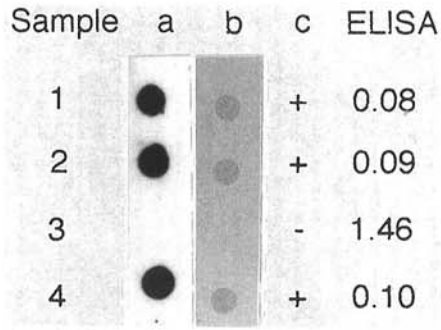

Fig. 5. Detection of Indian peanut clump virus (IPCV) infection in extracts of peanut seeds. Known quantities of total nucleic acids extracted from peanut seeds were hybridized with the digoxigenin-labeled probe and developed with either chemiluminescent (lane a) or chromogenic substrate (lane b). Samples of the same seeds were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of IPCV-H (from Hyderabad); the seeds were germinated, and the resulting seedlings were assayed for infection with ELISA (lane c; + indicates infection of seedlings). In this assay, high absorbance values indicate that no conjugate was bound; low values mean that the conjugate was bound, i.e., IPCV was present.

Although probe 4 hybridized well with RNA from IPCV and PCV, there was no hybridization with RNA of the other furoviruses: SBWMV, BNYVV, or PMTV (data not shown).

Comparison of sensitivities of radioactive and nonradioactive probes. Blots containing dilution series of RNA extracted from IPCV particles were probed with probe 4 labeled with either ^{32}P-dATP or DIG-dUTP (DIG-labeled probes). Hybridization was detected by autoradiography or by using either chemiluminescent or chromogenic substrates for the alkaline phosphatase conjugated anti-DIG. Assays using the DIG-labeled probes detected less than 10 pg of RNA (Fig. 3), which was a sensitivity comparable with that obtained using the ^{32}P-labeled probes. Chromogenic detection of DIG was similar in sensitivity to chemiluminescent detection. When extracts from various plant tissues were probed, a signal was obtained only with extracts from infected plants (Figs. 4 and 5). However, when extracts were made from peanut leaf samples, a weak background signal was detectable. This was eliminated when the extracts were diluted a further 10-fold. This problem has also been reported by others and found to be avoidable by using chemiluminescent detection (2,4).

Versatility of the broad-spectrum probe. To test the use of probe 4 in the detection of IPCV infection in field samples, dot blots were prepared of total nucleic acids from leaf and root material of several weed species that occur in clump-affected fields and of wheat plants grown in fields previously containing clump-infected peanuts. The blots were probed with DIG-labeled probe 4. Extracts of weeds taken from fields containing IPCV-infected peanut plants reacted with the probe (Fig. 4A, lane I), but samples from similar plants from adjacent, disease-free areas in the same fields did not (Fig. 4A, lane H). Extracts of some of the wheat plants hybridized with probe 4 (Fig. 4B; A1, A2, and B1), but others (Fig. 4B, B2) did not. Plant extracts that hybridized with the probe were shown to contain particles of IPCV by immunosorbent electron microscopy, ELISA, and inoculation to test plants.

Detection of IPCV in peanut seed. As IPCV is seedborne, experiments were done to see if IPCV could be detected in seeds. Samples were taken from several seeds and assayed as RNA extracts by dot-blot hybridization using DIG-labeled probe 4 and as buffer extracts by ELISA. The remainder of each seed was then germinated, and the resulting seedling was assayed for the presence of IPCV by ELISA. DIG was detected using either chemiluminescent or chromogenic substrates.

In these tests, chemiluminescent detection gave a stronger signal than did chromogenic detection (Fig. 5). There was a direct correlation between obtaining a positive hybridization signal and

the detection of IPCV by either ELISA (a low value signifying infection) or grow-out tests. There was no nonspecific reaction with uninfected seed samples. Seed samples extracted in buffers lacking LiCl did not give reproducible results in dot-blot assays.

DISCUSSION

cDNA probes were obtained that detected H serotype, but not the L or T serotypes (probe 1), or that could readily detect all three IPCV serotypes and PCV (probe 4). A particular advantage of probe 4 when used in disease surveys is that it should be capable of detecting novel serotypes of IPCV for which no antiserum is currently available. Moreover, the recent demonstration that IPCV causes a disease in wheat in India (3) and that PCV infects sorghum in Africa (6) suggests that there may well be viruses in cereal crops and weed species that are related to IPCV and PCV, but that are undetectable by the currently available antisera.

Probe 4 has the added advantages of being highly sensitive and applicable to the detection of IPCV in many plant species. The sensitivity of detection of IPCV RNA was comparable with that reported (1,4) for the detection of peanut stripe virus (PStV). Dietzgen et al. (4) reported that the detection of PStV in peanut seed using nonradioactive methods was difficult, but we found that, whereas detection of IPCV was possible using probe 4, the use of probes 1, 2, or 3, or the omission of the LiCl from the extraction protocol when using any probe gave poor results. The 3′-terminal 276 nucleotides of PCV RNA-1 (8) is 95% identical in sequence to the corresponding region of IPCV RNA-1, and the two RNAs of IPCV are almost identical in this region (J. S. Miller, *unpublished data*). This similarity means that a sample of IPCV RNA has, in effect, twice the template concentration for probe 4 that it has for the other probes. The ability of probe 4 to hybridize to both RNA molecules of different serotypes of IPCV and PCV may explain its superior performance over the other probes.

In summary, the assays we described for infection by IPCV should prove valuable in quarantine screening, diagnosis, and field surveying for IPCV, as well as related viruses such as PCV.

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