Detection of Two Viruses in Peanut Seeds by Complementary DNA Hybridization Tests

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ABSTRACT

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Dot blot hybridization was applied to the detection of peanut mottle virus (PMV) and peanut stripe virus (PStV) in peanut seeds. Both viruses can be detected readily in 1 mg of infected seed tissue and when extracts from seeds have been diluted 1/62,500 with buffer. One part of an infected seed can be reliably detected when it is mixed with 99 parts of healthy seeds. This sensitivity is 8–10 times greater than that achieved by use of the enzyme-linked immunosorbent assay. The stringent hybridization conditions of this procedure differentiated PMV and PStV in infected peanut seeds, even though the two viruses share considerable nucleotide sequence homology.

Peanut mottle virus (PMV) and peanut stripe virus (PStV) are common causal agents of mottling diseases of peanut (*Arachis hypogaea* L.). PMV occurs worldwide (3) and PStV occurs in Southeast Asia (12) and the United States (5). Both viruses are potyviruses, seed-transmitted in peanut, and transmitted by aphids in a nonpersistent manner. Thus far, resistance to either PMV or PStV has not been found in commercial or agronomically acceptable peanut. In the United States, where the source of primary inoculum of PMV is peanut seeds, the use of virus-free seeds appears to be a reasonable control practice (9). We speculate that the practice would be similarly effective for PStV.

Screening for virus-free seeds is labor-intensive, expensive, and time-consuming. Biological assays, such as infectivity and growing-out tests, are impractical, except for the evaluation of small seed lots of a few important peanut genotypes. The enzyme-linked immunosorbent assay (ELISA) has proved to be sensitive, inexpensive, and reliable for the detection of PMV and PStV in peanut seeds (2,7). One limiting factor is that only a relatively few seeds (10–12) can be combined for accuracy in one ELISA sample because additional seeds reduce the absorbance values to questionable levels (2,7).

Dot hybridization has been successfully used to screen for disease resistance in a potato breeding program (1). The method is sensitive, specific, rapid, and relatively easy to perform. It can be designed to detect a specific virus, a strain of a virus, or viruses within a group. In this study, the method was adapted to detect PMV- and PStV-infected peanut seeds. The

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sensitivity of the test for detecting virus in simulated contaminated seed lots was also determined.

MATERIALS AND METHODS

ELISA to select peanut seeds. Peanut seeds were tested for PMV and PStV by double antibody sandwich ELISA (4). Approximately 20 mg of cotyledonary tissue, removed from the end opposite the radicle, was pulverized in 0.5 ml of 20 mM potassium phosphate buffer (pH 7.3) containing 150 mM sodium chloride, 30 mM potassium chloride, 0.2% (w/v) polyvinylpyrrolidone (M_r = 40,000), 0.05% (v/v) Tween 20, and 10 mM sodium diethyldithiocarbamate (DIECA). The peanut seed extracts (200 μl) were then placed in microtiter plate wells previously coated with appropriate immunoglobulin. Other ELISA steps followed the method described by Clark and Adams (4). The enzyme and substrate used in the system were alkaline phosphatase and *p*-nitrophenyl phosphate, respectively.

Biological assay of virus. For suspected PMV-infected seeds, approximately 30 mg of cotyledonary peanut tissue was pulverized in 2 ml of 0.01 M potassium phosphate buffer (pH 8.0) containing 0.2% DIECA, 0.2% sodium bisulfite, and 1% Celite. Extract was rubbed onto primary leaves of *Phaseolus vulgaris* L. 'Topcrop,' a necrotic local lesion host. Extracts from PStV-infected seeds were prepared similarly and rubbed onto fully expanded leaves of *Chenopodium album* L. subsp. *amaranticolor* Coste & Reyn. (chlorotic local lesions) and young leaves of *Lupine albus* L. (systemic mottle).

Preparation of nucleic acid from healthy peanut seeds. Chopped peanut tissue from whole seeds (negative for virus by ELISA) was homogenized in extraction buffer (100 mM Tris buffer [pH 7.6] containing 1% [w/v] sodium dodecyl sulfate [SDS]) with a homogenizer (Tissumizer Model TR-10, Tekmar, Cincinnati, OH) for 1 min. The homogenate was clarified by centrifugation at 4 C at 10,000 *g* for 10 min. The aqueous phase was phenol-extracted twice and precipitated twice with three volumes of ethanol containing 250 mM sodium acetate.

Preparation of nucleic acid from test peanut seeds for dot hybridization. Peanut cotyledons were pulverized in extraction buffer in RNase-free mortars that had been stored at −80 C. The tissue-to-buffer ratio was usually 1:10. With small quantities of tissue (1–10 mg), however, it was necessary to alter the ratio to 1:100 or higher. Cellular debris was removed by centrifugation at 4 C at 10,000 *g* for 10 min. The aqueous phase was collected and denatured in 7% formaldehyde at 65 C for 15 min. The final dilution of the sample at this stage was 1:20. Low-speed centrifugation was repeated, and, with the aid of a hybri-dot manifold (Bethesda Research Laboratories, Gaithersburg, MD), 200-μl samples were applied to nitrocellulose filters (Bio-Rad, Richmond, CA) that had been presoaked for 10 min in sterile deionized distilled water, then equilibrated with 10× SSC (3 M sodium chloride and 300 mM sodium citrate) overnight. The filters were rinsed on the untreated side with 6× SSC, air-dried, baked at 85 C for 2 hr in a vacuum oven, and stored at 4 C prior to prehybridization.

Purification of viruses and RNAs. PMV and PStV were maintained in *Pisum sativum* L. 'Little Marvel' and *L. albus*, respectively. Both viruses were purified by the method of Demski et al (6). RNAs were isolated from purified viruses by the method previously described for a potyvirus (17).

Preparation of radiolabeled probes. Complementary DNA (cDNA) to PMV was synthesized using the RNA from PMV-M (type strain), which is the predominant strain in Georgia. PMV-M shares significant sequence homology with other PMV isolates (15). The cDNA to PStV was synthesized using the RNA from the stripe isolate of PStV as template. ^{32}P-labeled cDNA was synthesized by the random primer method (16), with minor modifications. Placental ribonuclease inhibitor (Sigma Chemical Company, St. Louis, MO) was added to the reaction mixture and sodium pyrophosphate was substituted for actinomycin D. Deoxycytidine 5′-triphosphate, tetra-(triethylammonium) salt, (α-^{32}P) of 3,000 Ci/mmol specific activity was obtained from NEN Research Products, Boston, MA.

Dot hybridization. The Owens and Diener method (11) was modified. The filters with samples were prehybridized for 1 hr at 42 C in 5 ml of 10 mM sodium cacodylate buffer (pH 7.0) containing 180 mM sodium chloride, 1 mM disodium ethylenediaminetetraacetic acid (EDTA), 0.1% (w/v) glycine, 40% (v/v) deionized formamide, and 400 ng/ml of purified yeast RNA or nucleic acid from healthy peanut seeds by the method described above. These filters were either used directly in the hybridization step or stored at 4 C.

The hybridization step was carried out at 55 C for at least 24 hr in 3 ml of 10 mM sodium cacodylate buffer (pH 7.0) containing 180 mM sodium chloride, 1 mM EDTA, 0.1% (w/v) SDS, 10% (w/v) sodium dextran sulfate, 40% (v/v) deionized formamide, and 400 ng/ml of either purified yeast RNA or nucleic acid from healthy peanut seeds and ^{32}P-labeled cDNA ($1–1.5 \times 10^6$ cpm/blot). After hybridization, the filters were washed twice in 2× SSC and 0.1% (w/v) SDS for 5 min at room temperature and twice again at 55 C. Two final washes were carried out in 0.1× SSC and 0.1% (w/v) SDS for 15 min at 55 C. Washed filters were exposed to XAR-5 X-Ray film (Eastman Kodak Company, Rochester, NY) at −80 C with a Cronex Lightning Plus intensifying screen (NEN Research Products).

RESULTS

ELISA detection of virus in peanut seeds. PMV-infected seeds were divided into two groups according to the ELISA absorbance readings: one with an intermediate reading range (A_{410nm} = 0.4–1.0) and another with a high reading range (A_{410nm} = >1.0). The same criterion was used to divide PStV-infected seeds into three groups; the intermediate and high ranges were the same as those with PMV-infected seeds and a low reading range group (A_{410nm} = 0.1–0.14) was added. Seeds from the latter group were included in some preliminary tests because the absorbance readings were two or more times greater than that of the healthy peanut control (range from 0.00 to 0.05). When the biological assay was used, however, no chlorotic local lesions were observed on *C. a.* subsp. *amaranticolor* and no systemic symptoms appeared on lupine. Thus, little or no virus was detected in the seeds in the low reading range group. Preliminary dot blot hybridization tests also gave negative results, and the seeds were dropped from further tests. Seeds infected with either PMV or PStV from each absorbance range group (intermediate and high) and yielding correlative numbers of lesions (more lesions with seeds from the high group than with seeds from the intermediate group) on the assay hosts were selected and processed for the dot blot hybridization.

cDNA detection of virus. Samples of 1 and 10 mg (compared with the standard sample size of 20 mg) of cotyledonary tissue from individual seeds (high ELISA reading range) infected with PMV or PStV were tested by ELISA and by dot blot hybridization. The cDNA probes to PMV and PStV each readily detected homologous viral RNA in 1- and 10-mg samples, whereas ELISA values from the same tissue were inconsistent (low, questionable absorbance values) with both 1- and 10-mg samples.

When RNA samples were diluted (six dilutions from 1:20 to 1:62,500) in nucleic acid extraction buffer, each cDNA probe detected homologous viral RNA from PMV- and PStV-infected seeds at all levels (Fig. 1).

Improvement of peanut seed preparation. One problem encountered when peanut seeds were pulverized and prepared for dot blot loading was viscosity of the seed extracts. When the dilutions were 1:20 or less, the tissue suspension clogged the nitrocellulose filters and most of the preparations had to be discarded. Attempts to reduce the viscosity with treatments of phenol, chloroform, and other organic solvents greatly reduced the sensitivity of the test. Low-speed centrifugation prior to

formaldehyde denaturation reduced viscosity to an acceptable level and did not affect the sensitivity of the test with either PStV or PMV (*data not shown*).

Detection of PMV and PStV in seed lots. A small portion (1–8 mg) of PMV-or PStV-infected peanut seed tissue was mixed with 49, 99, 199, and 399 parts of healthy peanut seed tissue (negative by ELISA and infectivity tests). All PMV-infected (Fig. 2) and PStV-infected (Fig. 3) samples (both viruses in the intermediate and high ELISA reading groups) could be readily detected at the 1:100 mixture level. Some samples, particularly those of PMV with high ELISA readings, could be readily detected at higher dilutions (Fig. 2).

The concentration of PMV and PStV in peanut seeds was estimated to be 50–100 μg/g of tissue, on the basis of the amount of seed tissue used per dot and comparison of those dots with the density and size of the standard (purified) RNA dots (Fig. 2).

Specificity of the test. PMV cDNA did not hybridize with samples of PStV-infected seeds (Fig. 2), and vice versa (Fig. 3). Purified PMV and PStV RNAs at a concentration of 1 ng/dot (readily detected with homologous cDNA) also were not detected by heterologous cDNA probes (Figs. 2 and 3). Both PMV and PStV cDNAs failed to hybridize with heterologous RNAs, even at 1 μg of RNA per dot.

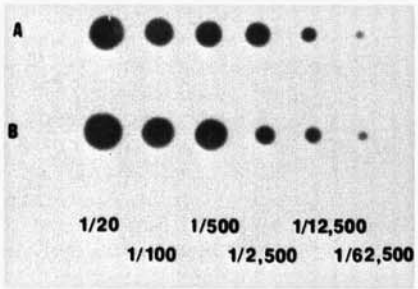

Fig. 1. Detection of viruses in various dilutions of extracts of peanut seeds by dot blot hybridization. Infected peanut seeds with ELISA readings above 1.0 absorbance units were extracted and diluted 1/20, 1/100, 1/500, 1/2,500, 1/12,500, and 1/62,500 with extraction buffer. Row A is seed preparations infected with peanut mottle virus and row B is seed preparations infected with peanut stripe virus. The membrane filters were treated with homologous cDNAs for 24 hr. Exposure time of the autoradiograph was 8 hr.

DISCUSSION

The dot blot hybridization technique can be used to detect peanut seeds infected with PMV and PStV, either in single seeds or in mixtures of infected and healthy seed tissue. The sensitivity of the test is 8–10 times greater than that of the ELISA technique now being used for routine screening of peanut seeds (2,7). Using double antibody sandwich ELISA, Demski and Warwick (7) showed that 10 peanut seeds can be combined for reliable detection of one PStV-infected seed. Bharathan et al (2) presented evidence that 12 seeds can be grouped for detection of one PMV-infected seed. Our results with dot blot hybridization indicate that one part of seed tissue infected with either PMV or PStV can be reliably detected when combined with about 100 parts of uninfected seed tissue. The dilution end point for extracts from a single PMV-infected seed was reported as 1/3,600 by ELISA (2), 17 times less than the 1/62,500 dilution for both PMV and PStV observed in this experiment. We believe the sensitivity of the dot blot method has not been developed to the limit yet. The limitation is the vicosity of the peanut seed extracts. If the problem can be totally eliminated and dilutions of less than 1/20 can be filtered through nitrocellulose, the sensitivity of the test probably can be improved.

The sensitivity of the dot blot hybridization test can be improved by prehybridization of membrane filters with heterologous RNA. Flores (8) reported reduction of nonspecific background in the detection of citrus exocortis viroid in clarified sap by prehybridizing filters with fractions of nucleic acid from healthy plants. Every filter in our present study was prehybridized with purified yeast RNA or purified RNA from healthy peanut seeds, and nonspecific background was minimal.

Besides sensitivity, rapidity, and simplicity, dot blot hybridization can be highly specific. PMV cDNA does not hybridize with PStV from infected seeds, and vice versa, even though the two viruses do share 60% nucleotide sequence homology when hybridization is performed under stringent conditions (15). ELISA is similarly specific in differentiating PMV and PStV in infected seeds (J. W. Demski, *personal communication*).

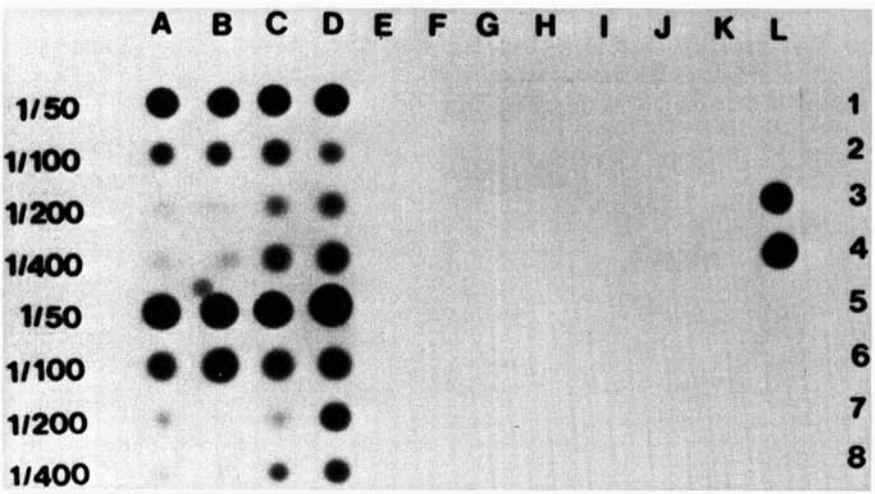

Fig. 2. Test for peanut seeds infected with peanut mottle virus (PMV) and peanut stripe virus (PStV) by dot blot hybridization with PMV cDNA probe (10^6 cpm/blot). Columns A–D are extracts from PMV-infected seeds; columns F–J are from PStV-infected seeds. ELISA readings (A_{405nm}) of seeds in each column are: A = 0.53, B = 0.75, C = 1.11, D = 1.75, F = 0.48, G = 0.61, H = 0.64, I = 1.19, J = 1.53, and E and K = no seed tissue. One part of tissue from PMV- or PStV-infected seeds was mixed with 49 (rows 1 and 5), 99 (rows 2 and 6), 199 (rows 3 and 7), and 399 (rows 4 and 8) parts of tissue from healthy peanut seeds. Column L contains controls: rows 1 and 2 = 0.1 ng PMV RNA, rows 3 and 4 = 1 ng PMV RNA, rows 5 and 6 = 1 ng PStV RNA, and rows 7 and 8 = healthy peanut seed preparations (faint but detectable spots were observed on rows 1 and 2 of the original autoradiograph).

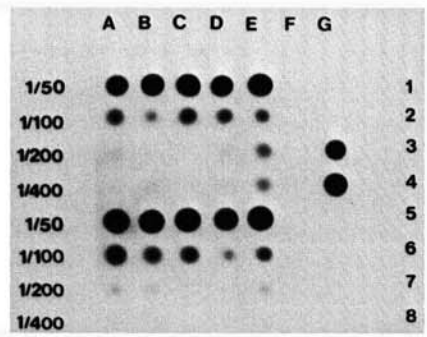

Fig. 3. Test for peanut seeds infected with peanut stripe virus (PStV) by dot blot hybridization with PStV cDNA probe (10^6 cpm/blot). ELISA readings (A_{405nm}) of seeds in each column are: A = 0.48, B = 0.61, C = 0.64, D = 1.19, E = 1.53, and F = no seed tissue. One part of tissue from PStV-infected seeds was mixed with 49 (rows 1 and 5), 99 (rows 2 and 6), 199 (rows 3 and 7), and 399 (rows 4 and 8) parts of tissue from healthy seeds. Column G contains controls: rows 1 and 2 = 0.1 ng of PStV RNA, rows 3 and 4 = 1 ng of PStV RNA, rows 5 and 6 = 1 ng of peanut mottle virus RNA, and rows 7 and 8 = healthy peanut seed preparations (faint but detectable spots were observed on rows 1 and 2 of the original autoradiograph).

When stringency of the hybridization reaction is carefully controlled and when cDNA libraries of unique sequences of virus strains are available, dot blot hybridization has the potential to provide a rapid and convenient approach to identification of strains of viruses. An example is citrus tristeza virus (CTV). Strains of CTV are serologically indistinguishable, and identification of these strains relies on biological indexing. With dot blot hybridization and a strain-specific probe, Rosner et al (14) were able to identify a specific isolate that causes severe symptoms in citrus.

New approaches to dot blot hybridization are still possible. cDNA libraries of PMV and PStV, not available at the time of this study, would greatly facilitate the assay. A homogenous probe can be synthesized using the nick translation technique. This will also provide the opportunity to produce a nonradiolabeled probe. The disadvantage of using the ^{32}P-labeled probe is the inconvenience of handling radioactive waste and the short-lived nature of the probe. The development and improvement of longer half-lived probes, such as those labeled with ^{35}S nucleotide, and nonradioactive probes, such as those labeled with biotin (10) or DNA-binding protein (13), should also be investigated.

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