Journal of Virological Methods, 23 (1989) 299–312
Elsevier

JVM 00841

Nonradioactive, photobiotin-labelled DNA probes for routine diagnosis of viroids in plant extracts

James L. McInnes, Nuredin Habili and Robert H. Symons

Commonwealth Special Research Centre for Gene Technology, Department of Biochemistry, University of Adelaide, Adelaide, South Australia, Australia

(Accepted 17 October, 1988)

Summary

Avocado sunblotch viroid (ASBV), coconut cadang cadang viroid (CCCV), chrysanthemum stunt viroid (CSV) and potato spindle tuber viroid (PSTV) were detected in plant extracts by dot-blot hybridization using nonradioactive photobiotin-labelled nucleic acid probes. Recombinant DNA probes, containing full-length monomer viroid inserts in the plasmid vectors pSP64 or pUC9, were biotinylated with photobiotin and used as sonicated double-stranded DNA fragments. Using fresh leaf material, a general method (suitably modified for avocado tissue) was developed for the rapid preparation of purified nucleic acid extracts. Plant extracts from a range of field samples were spotted onto nitrocellulose, subjected to hybridization and the biotin-labelled DNA bound to the target nucleic acid was detected with an avidin-alkaline phosphatase conjugate. Under the stated hybridization and washing conditions, each individual viroid probe was specific. Each viroid was readily detected with a sensitivity similar to that obtained with the same (or a like) probe labelled with ^{32}P. Healthy plant extracts gave colourless spots.

Viroid, avocado sunblotch; Viroid, chrysanthemum stunt; Viroid, coconut cadang cadang; Viroid, potato spindle tuber; DNA probe, photobiotin-labelled; Hybridization, dot-blot for diagnosis

Correspondence to: R.H. Symons, Commonwealth Special Research Centre for Gene Technology, Department of Biochemistry, University of Adelaide, Adelaide. S.A. 5000, Australia.

Introduction

Viroids, the smallest known independently replicating pathogens, occur in a wide range of economically important horticultural and agricultural crops (Diener, 1987; Keese and Symons, 1987). Since they lack the antigenic coat protein characteristic of viruses, viroids cannot be detected by immunological approaches such as the enzyme-linked immunosorbent assay (ELISA) (Owens and Diener, 1984; Symons, 1984). Traditionally, diagnostic methods for viroids have been biological in nature, involving sap and graft inoculation of indicator plants. These tests, while being sensitive and indicative of the infectious nature of the causal agent, have proved inefficient because of their labour-intensive nature and the long incubation periods (e.g. up to 2 years in the case of avocado sunblotch viroid [ASBV]) needed for symptom development. Alternative approaches, e.g. two-dimensional polyacrylamide gel electrophoresis on plant extracts (Schumacher et al., 1983), tend to suffer from sensitivity problems and do not provide a positive identification of the viroid.

Detection methods for viroids based on nucleic acid hybridization have been employed in recent years and have proved extremely reliable and sensitive (e.g., Barker et al., 1985; Lakshman et al., 1986). The dot-blot hybridization assay (Thomas, 1983; Meinkoth and Wahl, 1984; Anderson and Young, 1985) involving the use of radioactive probes has become widely used in many countries for the routine detection of a range of viroids (McInnes and Symons, 1989). A major advantage with dot-blot hybridization is the sensitivity of detection; viroids can be detected in the low picogram range in many plant extracts.

Recently, we developed a nonradioactive DNA probe for routine diagnosis of barley yellow dwarf virus (BYDV) in plant extracts (Habili et al., 1987). This probe was labelled non-isotopically by a simple chemical procedure involving a photoactivatable analogue of biotin, called Photobiotin™ (Forster et al., 1985; McInnes et al., 1988 a,b). This paper describes a general dot-blot hybridization protocol using photobiotin-labelled recombinant DNA probes for the specific detection in plant extracts of four important viroids, viz: ASBV, coconut cadang cadang viroid (CCCV), chrysanthemum stunt viroid (CSV) and potato spindle tuber viroid (PSTV). The sensitivity and specificity of detection of these probes is comparable to ^{32}P-labelled DNA probes used under the same conditions.

Materials and Methods

Source of healthy and infected plant material

Healthy avocado leaf material from field trees was obtained from M. Sedgley, Waite Agricultural Research Institute, University of Adelaide, S.A. Infected avocado leaves were obtained from plants inoculated with ASBV and maintained at about 25°C and 16 h light in a growth room. Healthy and CCCV-infected coconut palm leaf material was obtained from J. Randles and D. Hanold, Waite Agricultural Research Institute, University of Adelaide. These samples included leaf ma-

terial from field palms grown in the Solomon Islands and the Philippines. Healthy coconut leaf material was also obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO), Darwin, Northern Territory (via J. Possingham). Healthy and CCCV-infected oil palm leaves were obtained from plants maintained in the growth room.

Healthy and CSV-infected chrysanthemum leaf material was obtained from locally grown plants and from R.A.C. Jones, W.A. Department of Agriculture, Perth, W.A. Healthy potato leaf material was obtained from S. Holland, Plant Research Institute, Burnley, Victoria and C. Williams, S.A. Department of Agriculture, Lenswood, S.A. Healthy and PSTV-infected tomato leaves were obtained from uninoculated and PSTV-inoculated plants (c.v. Grosse Lisse) maintained in the growth room.

Extraction buffers

AMES buffer is 0.5 M sodium acetate, pH 6.0, 10 mM $MgCl_2$, 3.0% (w/v) SDS and 20% (v/v) ethanol (Laulhere and Rozier, 1976). STE buffer is 50 mM Tris-HCl, pH 7.2, 0.1 M NaCl, 1 mM EDTA.

Preparation of chrysanthemum, coconut palm, oil palm, potato and tomato extracts

A nucleic acid extract was prepared from freshly harvested healthy and viroid-infected plant tissue by the following procedure. Intact leaves were rinsed with glass-distilled water and crushed between the rollers of a sap extractor (Erich Pollahne, F.R.G.). For each gram of tissue, 5 ml AMES buffer containing 1% (v/v) 2-mercaptoethanol was added dropwise to the tissue attached to the rollers. The slurry was collected in 50 ml centrifuge tubes, vortexed for 1 min with 2.5 ml water-saturated phenol and 2.5 ml chloroform, and placed on ice for 10 min. The aqueous phase was recovered by spinning the mixture at 10000 × *g* at 4°C for 20 min. The nucleic acids were precipitated by the addition of 2.5 vol. chilled ethanol to all or part (0.4 ml) of the aqueous phase (50 ml Corex or 1.5 ml Eppendorf tubes), followed by incubation at −20°C for 1 h. After centrifugation at 10000 × *g* at 4°C for 20 min, the nucleic acid pellet was washed carefully with cold 70% (v/v) ethanol, recentrifuged as above for 10 min, dried under vacuum, resuspended in 0.1 mM EDTA (100–200 µl/g tissue), and frozen at −20°C. After thawing, the extract was centrifuged at 10000 × *g* at 4°C for 10 min and the supernatant was removed and stored at −20°C.

Preparation of avocado extracts

A nucleic acid extract was prepared from freshly harvested healthy and ASBV-infected plant tissue by the following procedure. In view of the reported marked differences in the levels of ASBV found in leaf extracts from separate avocado trees and from leaves taken from different parts of the same tree (Allen and Dale, 1981; Palukaitis et al., 1981), it is advisable to assay a sample of pooled leaves, harvested from various positions in each tree.

Intact avocado leaves were rinsed initially with glass-distilled water and then with extraction buffer (AMES buffer containing 1% (v/v) 2-mercaptoethanol and 1.2%

(w/v) sodium sulphite). Several drops of the extraction buffer were added to the rotating rollers of the sap extractor and the leaves were then crushed as above (5 ml extraction buffer/g tissue). Water-saturated phenol (2.5 ml) was added to the leaf material remaining on the extractor rollers and the slurry collected. Chloroform (2.5 ml) was added, the slurry was shaken for 1 min and placed on ice for 10 min. The remainder of the procedure was identical to that stated above for chrysanthemum, coconut palm, oil palm, tomato and potato tissue.

CF11-cellulose purification of extracts from woody perennials

The procedure used was adapted from Allen and Dale (1981) and Rezaian et al., (1988) and involved the selective adsorption of nucleic acids on Whatman CF11-cellulose (Cat. No. 4021-050) in STE buffer containing 35% (v/v) ethanol, and their subsequent elution with 0.1 mM EDTA containing no ethanol.

CF11-cellulose purification began after the ethanol precipitation step described previously (see: Preparation of chrysanthemum, coconut palm, oil palm, potato and tomato extracts). The wet nucleic acid pellet resulting from extraction of 1 g avocado, coconut or oil palm tissue was resuspended in 4 ml of STE buffer containing 35% (v/v) ethanol. After the addition of 0.1 g of dry CF11-cellulose, the mixture was shaken for 10 min at room temperature, centrifuged at 3000 × *g* at 4°C for 2 min and the supernatant discarded. The cellulose containing the bound nucleic acids was further washed two or three times (30 s vortexings) with 3 ml aliquots of chilled STE buffer containing 35% (v/v) ethanol. Nucleic acids were then eluted with 2 × 1 ml aliquots of 0.1 mM EDTA and precipitated by the addition of 0.1 vol. 3 M sodium acetate and 2.5 vol. chilled ethanol, followed by incubation at −20°C for 1 h. After centrifugation at 10000 × *g* at 4°C for 20 min, the nucleic acid pellet was washed carefully with 70% (v/v) ethanol, recentrifuged as above for 10 min, and dried under vacuum. The nucleic acid pellet was resuspended in 0.1 mM EDTA (100–200 µl/g tissue) and frozen at −20°C.

Viroid cDNA clones

Recombinant DNA clones of ASBV and CCCV, originally cloned into the single-strand M13mp93 phage DNA vector (Barker et al., 1985), were recloned into the *Bam*H1 site of the plasmid vector pSP64 (Hutchins et al., 1985). A recombinant DNA clone containing a full-length monomer insert (353 residues) of the Beltsville, U.S.A., strain of CSV in the plasmid pUC9, was a gift from R.A. Owens, U.S. Department of Agriculture, Beltsville, Maryland, U.S.A. The plasmid clone pAV401 (Van Wezenbeek et al., 1982), containing a complete cDNA copy of PSTV cloned into plasmid pBR322, was a gift from P. Vos, The Agricultural University, Wageningen, The Netherlands. This latter clone was recloned into the *Bam*H1 site of pSP64. All plasmid clones were propagated in *E. coli* MC1061.

Preparation of ^{32}P- and photobiotin-labelled DNA probes

Recombinant plasmid SP6 DNA (ASBV, CCCV, PSTV) and pUC DNA (CSV) were prepared according to a modified alkaline lysis protocol (Maniatis et al., 1982) and labelled with Photobiotin™ (Bresatec Ltd., GPO Box 498, Adelaide, S.A.

5001, Australia) as described in Forster et al. (1985), McInnes et al. (1988a,b), and in the protocol supplied by Bresatec. These biotinylated probes were sonicated as described in Habili et al. (1987) and stored in 0.1 mM EDTA at −20°C.

^{32}P-cDNA M13 single-strand ASBV and CCCV probes (specific activity 3×10^9 cpm/μg DNA) were prepared by the downstream primer approach of Barker et al. (1985), dissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1% (w/v) SDS, 5 mM 2-mercaptoethanol, and stored at −20°C. Recombinant plasmid SP6 DNA (PSTV) and pUC DNA (CSV) were labelled with α-[^{32}P]dCTP by nick translation using a NTK-C kit (Bresatec) according to the manufacturer's instructions. The radiolabelled DNA was separated from protein by phenol/chloroform (1:1) extraction in the presence of carrier tRNA. Unincorporated ^{32}P-labelled nucleotide was removed from the aqueous solution by two successive ethanol precipitations. The [^{32}P]DNA probe (specific activity 7×10^7 cpm/μg DNA) was dissolved in the above buffer and stored at −20°C.

Formaldehyde treatment of chrysanthemum, coconut palm, oil palm, potato and tomato extracts

Prior to spotting onto nitrocellulose, plant extracts were denatured by formaldehyde using a modification of the original White and Bancroft (1982) procedure. One volume of plant extract was added to three volumes of 10 × SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate) containing 20% (w/v) formaldehyde; the mixture was then vortexed, incubated at 65°C for 15 min, and ice-cooled.

Formaldehyde treatment of avocado extracts

Extracts prepared from avocado leaves were treated with formaldehyde by a similar protocol to that stated above except that it was important to omit the heating step (65°C for 15 min) (see below).

Dot-blot hybridization procedure

The procedure used was adapted from Thomas (1983) and Barker et al. (1985). Prestamped sheets of nitrocellulose (Schleicher and Schuell, BA85, 6 × 6 mm squares) were soaked in glass-distilled water for 5 min and then in 20 × SSC for 30 min before drying under a lamp for 5 min. Samples (3 μl) of the formaldehyde-treated extracts were spotted onto the nitrocellulose filters which, after drying under a lamp for 5 min, were baked at 80°C in vacuo for 2 h. The filters were then transferred to a heat-sealable polythene bag and prehybridized at 42°C for 16 h in a prehybridization buffer consisting of 50% (v/v) deionized formamide, 5 × SSC, 50 mM sodium phosphate, pH 6.5, 0.25 mg/ml sonicated denatured salmon sperm DNA, 0.2% (w/v) SDS, 5 mM EDTA, and 0.2 mg/ml each of bovine serum albumin, Ficoll 400 (Pharmacia), and polyvinyl pyrrolidone (M_r 40 000). Hybridization was carried out at 55°C for 20–24 h in a mixture of 4 vol. prehybridization buffer and 1 vol. 50% (w/v) dextran sulphate.

Before commencement of hybridization the DNA probes were denatured as follows: photobiotin-labelled probes at approximately 8 ng/μl in 50 mM NaOH were incubated at room temperature for 10 min before adding directly to the hybridi-

zation buffer. [^{32}P]cDNA M13 single-strand probes and [^{32}P]DNA probes labelled by nick translation were heated in 50% (v/v) deionized formamide at 80°C for 3 min or 95°C for 10 min, respectively, before snap-cooling on ice. The final concentration of the photobiotin-labelled probe in hybridization buffer was 100 ng/ml and 0.5×10^6 cpm of ^{32}P-DNA probe was added per ml hybridization buffer. For each cm^2 of nitrocellulose filter, 0.1 ml of prehybridization and hybridization buffers were used.

Post-hybridization washing steps for both biotinylated- and ^{32}P-probes consisted of three washes in 2 × SSC, 0.1% (w/v) SDS for 15 min each at room temperature, followed by three washes in 0.1 × SSC, 0.1% (w/v) SDS for 20 min each at 55°C. Autoradiography of ^{32}P-labelled filters, suitably wrapped in thin polythene film, was at −70°C for 3–22 h using an intensifying screen.

Colorimetric detection of biotin-labelled probes

The method was adapted from that of Leary et al. (1983). Filters were placed in polythene bags and incubated in blocking buffer (0.1 M Tris-HCl, pH 7.5, 1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100 [TSMT buffer] containing 30 mg/ml bovine serum albumin) for 1 h at 42°C. The method of preparation for the blocking buffer is outlined below. The blocking buffer was removed, replaced with 1 μg/ml of avidin-alkaline phosphatase (Sigma, Cat. No. A-2527) in TSMT buffer and incubated for 15 min at room temperature with occasional gentle agitation. The filters were removed from the polythene bags and washed, with medium agitation, three times at room temperature for 20 min each in TSMT buffer followed by two washes at room temperature for 10 min each in 0.1 M Tris-HCl, pH 9.5, 1 M NaCl, 5 mM $MgCl_2$. (Thorough washing of the filters at this stage is necessary to minimize background coloration).

For colour development the filters were transferred to new polythene bags and incubated at room temperature in the dark with a substrate solution consisting of 0.1 M Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.30 mg/ml nitro blue tetrazolium (NBT) and 0.20 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP). (The substrate solution was prepared just prior to use by the addition of 20 μl NBT solution – 75 mg/ml in 70% *N,N*-dimethylformamide – and 20 μl BCIP solution (50 mg/ml in *N,N*-dimethylformamide) to 5 ml of the above buffer with gentle mixing.) The reaction was stopped after 1–2 h with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Filters were photographed while moist.

Preparation of blocking buffer

Bovine serum albumin (3 g, Fraction V, Sigma, Cat. No. A-4503) was dissolved in 70 ml sterile glass-distilled water in a screw-capped glass bottle and the resulting solution was adjusted to pH 3.0 with conc. HCl. After heating in a boiling water bath for 20 min, the solution was cooled to room temperature and adjusted to pH 7.5 with 10 M NaOH. During this pH adjustment, the solution briefly turns turbid, but readily clarifies as the pH nears neutrality; 10 ml TMT buffer (1.0 M Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.5% Triton X-100) and 5.8 g solid NaCl were added and the solution was adjusted to 100 ml with sterile glass-distilled water. The solution can be stored at 4°C for several months.

Results

Detection of ASBV

To test the ability of a photobiotin-labelled recombinant plasmid SP6 DNA probe in ASBV detection, extracts were prepared from freshly harvested tissue from several well-known varieties of avocado (Sharwil, Fuerte, Bacon, Hass and Reed), spotted onto nitrocellulose and probed by dot-blot hybridization. The results (Fig. 1A) indicated that each of the different avocado varieties was negative for ASBV infection (samples 1–6). No background colour was discernable with these healthy extracts which included one (sample 6) which had been prepared from avocado tissue frozen overnight (16 h). The biotinylated probe also correctly detected the known ASBV-infected extracts (samples 7–12). One of these extracts (sample 12) was from the same leaf material as sample 11 but had been prepared with an additional CF11-cellulose purification step (Materials and Methods). This extra decolorization step is recommended in two instances; first, for starting leaf material which has been stored at −20°C for long periods and, second, for extracts which, at the end of the recommended extraction procedure, are highly coloured. The strength of the signal obtained with this extract (sample 12; Fig. 1A) indicated that no loss of ASBV occurred during the CF11-cellulose step.

The above results were confirmed by the use of a single-strand ^{32}P-recombinant M13 phage DNA probe for ASBV which gave identical results (Fig. 1B). In addition, direct counting of the ^{32}P-label associated with the spotted extracts (samples 11,12; Fig. 1B) confirmed that no loss of ASBV occurred during the extra CF11-cellulose purification step.

Detection of CCCV

Healthy and infected coconut and oil palm tissues were extracted and assayed with a photobiotin-labelled recombinant plasmid SP6 DNA probe. The dot-blot hybridization results (Fig. 1D) showed that the biotinylated probe hybridized strongly to extracts of CCCV-infected coconut palms from the Philippines (samples 8,9) and to the known CCCV-infected oil palm extract (sample 2). No background colour was associated with healthy tissue (sample 1, oil palm; samples 3–7, coconut palm). As demonstrated with avocado tissue (previous section), an extract (sample 9) prepared from CCCV-infected coconut tissue and further purified on CF11-cellulose also showed excellent viroid recovery. The results were confirmed by the use of a single-strand ^{32}P-recombinant M13 phage DNA probe for CCCV which gave an identical pattern of results (Fig. 1E). Direct counting of the ^{32}P-label associated with the spotted extracts (samples 8,9; Fig. 1E) gave an approximate loss of 25% viroid after CF11-cellulose treatment.

Detection of CSV

Fig. 2A shows the results of a typical dot-blot hybridization study using a photobiotin-labelled recombinant plasmid pUC DNA probe for CSV detection. Leaf extracts were prepared from a range of field chrysanthemum samples, spotted onto nitrocellulose and probed in the normal fashion. Extracts prepared from infected

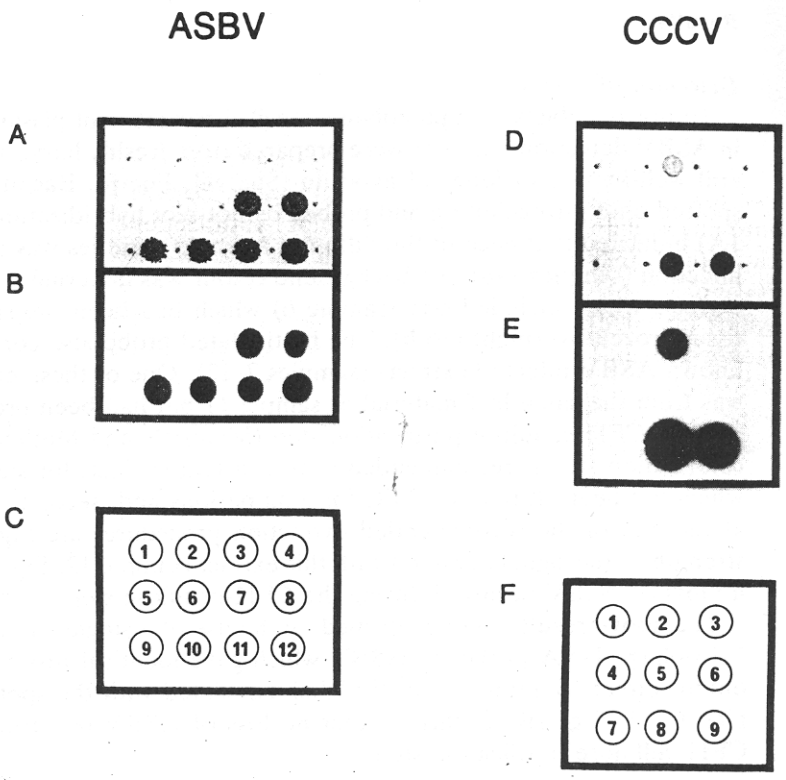

Fig. 1. Detection of ASBV and CCCV in plant extracts by dot-blot hybridization analysis. (A,B,C) *ASBV*: Extracts were prepared from healthy and infected avocado leaves (Materials and Methods), treated with formaldehyde without heating (Materials and Methods) and samples (3 μl) were spotted onto duplicate nitrocellulose membranes. Each spot contained extract from 4 mg of leaf tissue. Hybridization conditions were as in Materials and Methods. Samples 1–6 were obtained from field avocado trees: 1, c.v. Sharwil; 2, c.v. Fuerte; 3, c.v. Bacon; 4, c.v. Hass; 5, c.v. Reed; 6, c.v. Sharwil (tissue had been frozen overnight prior to extraction). Samples 7–12 were obtained from ASBV-inoculated avocado trees maintained in the growth room: 7–11, c.v. Hass; 12, c.v. Hass, CF11-cellulose treated. (A) Sonicated, photobiotin-labelled probe; colorimetric detection time was 2 h. (B) Single-stranded [^{32}P]-cDNA probe; autoradiography was for 3 h. (C) Location of samples on nitrocellulose membranes. (D,E,F) *CCCV*: Extracts were prepared from healthy and infected coconut and oil palm leaves (Materials and Methods), treated with formaldehyde (Materials and Methods) and samples (3 μl) were spotted onto duplicate nitrocellulose membranes. Each spot contained extract from 4 mg of leaf tissue. Hybridization conditions were as in Materials and Methods. Samples 1 and 2 were obtained from oil palms maintained in the growth room: Sample 3 was obtained from a coconut palm maintained under glasshouse conditions. Samples 4–9 were obtained from coconut palm field trees: 4, Solomon Islands; 5–7, Darwin, N.T., Australia; 8, Philippines; 9, Philippines, CF11-cellulose treated. (D) Sonicated, photobiotin-labelled probe: colorimetric detection time was 1 h. (E) Single-stranded [^{32}P]cDNA probe; autoradiography was for 6 h. (F) Location of samples on nitrocellulose membranes.

leaf tissue (samples 1,2,4,6–8) gave strong positive hybridization signals; extracts prepared from healthy plants (samples 3,5,9) showed no hybridization. An identical pattern of results was obtained (Fig. 2B) using the same recombinant DNA probe, labelled with ^{32}P by nick translation.

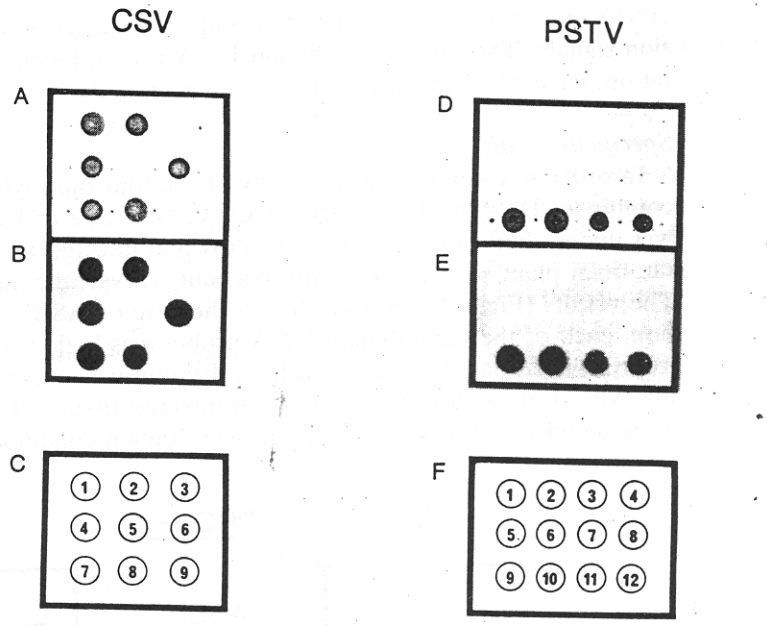

Fig. 2. Detection of CSV and PSTV in plant extracts by dot-blot hybridization analysis. (A,B,C) *CSV*: Extracts were prepared from healthy and infected chrysanthemum leaves (Materials and Methods) and samples (3 μl) were spotted directly without formaldehyde treatment onto duplicate nitrocellulose membranes. Each spot contained extract from 30 mg of leaf tissue. Hybridization conditions were as in Materials and Methods. Samples 1–7, field samples. Sample 8, known CSV-infected chrysanthemum tissue. Sample 9, known healthy chrysanthemum tissue. (A) Sonicated, photobiotin-labelled probe; colorimetric detection time was 1 h. (B) Nick-translated [^{32}P]DNA probe; autoradiography was for 19 h. (C) Location of samples on nitrocellulose membranes. (D,E,F) *PSTV*: Extracts were prepared from healthy and infected potato and tomato leaves (Materials and Methods), treated with formaldehyde (Materials and Methods) and samples (3 μl) were spotted onto duplicate nitrocellulose membranes. Each spot contained extract from 4 mg of leaf tissue. Hybridization conditions were as in Materials and Methods. Samples 1–4, field potatoes; 1, c.v. Coliban; 2, c.v. Promesse; 3 and 4, unknown cultivars. Samples 5, 7 and 8, field tomato plants (c.v. Grosse Lisse). Sample 6, glasshouse tomato plant (c.v. Grosse Lisse). Samples 9–12, PSTV-inoculated tomato plants (c.v. Grosse Lisse) maintained in the growth room. (D) Sonicated photobiotin-labelled probe; colorimetric detection time was 1 h. (E) Nick translated [^{32}P]DNA probe; autoradiography was for 18 h. (F) Location of samples on nitrocellulose membranes.

The photobiotin-labelled DNA probe could also be used for CSV detection in chrysanthemum extracts prepared according to Horst and Kawamoto (1980) (data not shown).

Detection of PSTV

We have also successfully employed photobiotin to produce a biotinylated recombinant plasmid SP6 DNA probe for the detection of PSTV in tomato and potato tissues using dot-blot hybridization. The results (Fig. 2D) showed that extracts prepared from healthy potato (samples 1–4) and healthy tomato (samples 5–8) plants showed no hybridization while extracts prepared from the infected

(PSTV-inoculated) tomato plants (samples 9–12) gave strong positive hybridization signals. The same recombinant DNA probe, labelled with ^{32}P by nick translation, gave an identical pattern of results (Fig. 2E).

Specificity of the probes

In order to examine the specificity of the four biotinylated double-stranded recombinant DNA probes (ASBV, CCCV, CSV and PSTV), a hybridization study was undertaken using, as target, healthy and infected tissue extracts from each of the main plant sources (avocado, coconut, chrysanthemum, potato and tomato). The results (Fig. 3A) showed that in the case of ASBV, CCCV and CSV detection, each of the recombinant DNA probes was highly specific under our hybridization conditions; i.e., no cross-hybridization was observed with any one particular viroid probe for the other viroid-infected tissues. This result was confirmed using identical extracts and identical hybridization conditions with ^{32}P-DNA probes

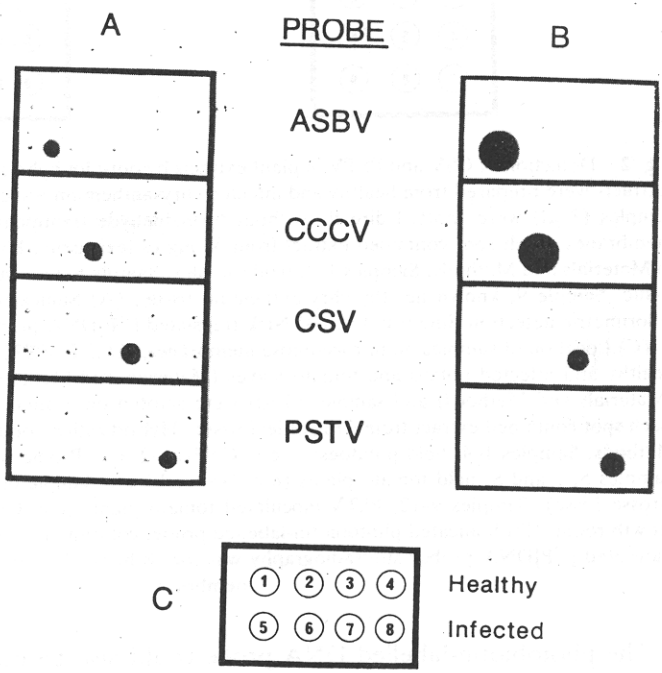

Fig. 3. Dot-blot cross-hybridization study for probe specificity. Extracts were prepared from healthy and infected tissue (Materials and Methods), treated with formaldehyde (Materials and Methods) and samples (3 µl) were spotted onto eight nitrocellulose membranes. Each spot contained extract from 4–8 mg of leaf tissue. Prehybridization, hybridization and washing conditions were as in Materials and Methods. Samples 1–4, healthy plant extracts; samples 5–8, infected plant extracts. 1 and 5, avocado; 2 and 6, coconut; 3 and 7, chrysanthemum; 4, potato; 8, PSTV-inoculated tomato. (A) Sonicated, photobiotin-labelled probes; colorimetric detection time was 1.5 h. (B) Single-stranded [^{32}P]cDNA (ASBV, CCCV) and nick translated ^{32}P-DNA (CSV, PSTV) probes. Autoradiography was for 22 h. (C) Location of samples on nitrocellulose membranes.

(Fig. 3B). However, the PSTV probe, either biotinylated or ^{32}P-labelled, did produce a very low cross-hybridization signal against CSV-infected tissue. These results cannot be seen in Fig. 3A and 3B owing to the extreme faintness of the recorded signals.

Discussion

Detection methods for viroids based on nucleic acid hybridization have become established worldwide (McInnes and Symons, 1988). There is an obvious need for such future routine diagnosis to be based on nonradioactive probe technology. In this context we have developed a rapid and sensitive non-isotopic method for the dot-blot detection of four viroids (ASBV, CCCV, CSV and PSTV) in plant extracts. We demonstrate for the first time the use of recombinant DNA probes labelled by photobiotin for the detection of viroids in field samples (Figs. 1 and 2). In the case of each viroid species, the degree of sensitivity achieved with the biotinylated probe was directly comparable to that obtained with a ^{32}P-labelled DNA probe, used under identical conditions (Figs. 1 and 2).

Initial studies aimed at the detection of PSTV in plant extracts with probes labelled by photobiotin established that formaldehyde treatment of plant extracts, the direct spotting of extracts onto nitrocellulose (rather than the use of a vacuum manifold) and the use of sonicated probes all contributed to an enhancement in sensitivity of the method (data not shown). These findings were in direct agreement with those obtained in a recent study involving photobiotin-labelled DNA probes for the diagnosis of BYDV in plant extracts (Habili et al., 1987).

Obviously, in establishing a nonradioactive diagnostic protocol for plant viroids based on a colorimetric detection scheme, it is essential to prepare non-coloured plant extracts. At the same time, any undue darkening or staining of the spotted extracts on the nitrocellulose filter during prehybridization and hybridization must be prevented. Unfortunately, in the case of woody perennial species such as avocado and coconut, coloured extracts can arise due to oxidation of certain phenolic compounds, proanthocyanidins, which are present in high levels in these tissues (Newbury and Possingham, 1977).

To avoid these problems we routinely used fresh tissue as starting material, included the reducing agent, 2-mercaptoethanol (1% v/v), in the extraction buffer and formaldehyde-treated the plant extracts prior to spotting onto nitrocellulose. Even so, avocado tissue proved extremely troublesome, giving rise to extracts which darkened considerably on nitrocellulose during prehybridization and hybridization. This effect was prevented by the inclusion of an additional reducing agent, sodium sulphite (1.2% w/v), in the extraction medium. The use of this chemical for similar purposes has been previously reported for coconut and potato tissue (Imperial et al., 1981; Smith and Banttari, 1987).

A further problem occurred with avocado tissue. Extracts prepared from ASBV-inoculated plants that had been heat-treated (65°C/15 min) in the presence of formaldehyde prior to spotting onto nitrocellulose routinely showed a loss of signal

strength after hybridization. This effect was rather variable; while the majority of extracts showed considerable signal loss, an occasional extract gave negligible loss. The exact nature of the factors involved has not been fully investigated but initial studies have shown the loss to be time-dependent with respect to the heat treatment and also preventable by CF11-cellulose purification (data not shown). Formaldehyde treatment of ASBV-infected plant extracts in the absence of heat, however, gave an approximate 2-fold increase in hybridization signal strength (data not shown). Hence, for the routine diagnosis of ASBV in plant extracts using the nonradioactive biotinylated probe, we recommend formaldehyde treatment of extracts without a heating step, prior to spotting onto nitrocellulose.

Several interesting observations occurred with fresh chrysanthemum tissue. The extracts routinely prepared were slightly coloured and, once spotted onto nitrocellulose, gave darkened spots. However, this colour completely disappeared during the pre-hybridization and hybridization steps, thus allowing colorimetric detection. Chrysanthemum extracts were also unique in that they could be applied directly to nitrocellulose without formaldehyde treatment, so necessary for the other plant material. This modification also enabled the spotting of a more concentrated extract to the nitrocellulose filter.

The use of frozen leaves (especially woody perennials) as starting material for plant extraction is not recommended for this nonradioactive diagnostic procedure. In our hands, the use of such material routinely resulted in coloured extracts. In this context, we were unsuccessful in attempts to prepare colorless extracts from avocado tissue stored frozen for long periods (e.g., 12 months or longer), although CF11-cellulose purification did remove the resulting colour (data not shown). The extraction of avocado leaf material stored briefly at −20°C (16 h, overnight) did, however, result in a colourless extract (Fig. 1A, sample 6). Long term storage of frozen CSV-infected chrysanthemum tissue should also be avoided. Our studies (unpublished observations) have indicated considerable loss of viroid with such frozen material as well as from stored, frozen chrysanthemum and tomato leaves infected with citrus exocortis viroid. In general, it is recommended that leaf material for diagnosis, irrespective of the plant source, be transported cooled and stored at 4°C prior to extraction. Storage of such material at 4°C for several days gave no coloured extracts or loss of viroid.

Finally, the non-isotopic dot-blot detection method outlined here may prove suitable for the analysis of other viroid species. In this context, we are hopeful of developing a photobiotin-labelled DNA probe for hop stunt viroid (HSV). Such a probe would prove extremely beneficial for the detection of HSV in Australian grapevine and hop cultivars (Koltunow et al., 1988).

Acknowledgements

This work was supported by a joint grant under the National Biotechnology Program Research Grants Scheme to the Department of Biochemistry, University of Adelaide, and the CSIRO Division of Plant Industry, Canberra. We thank Dr.

R. Owens and Dr. P. Vos for DNA clones, Dr. R.I.B. Francki for the use of glasshouse facilities, Dr. D. Hanold, Dr. S. Holland, Dr. R.A.C. Jones, Dr. J. Possingham, Dr. J. Randles, Dr. M. Sedgley and Dr. C. Williams for plant material, and Jenny Cassady and Tammy Edmonds for assistance.

References

Allen, R.N. and Dale, J.L. (1981) Application of rapid biochemical methods for detecting avocado sunblotch disease. Ann. Appl. Biol. 98, 451–461.

Anderson, M.L.M. and Young, B.D. (1985) Quantitative filter hybridization. In: B.D. Hames and S.J. Higgins (Eds.), Nucleic Acid Hybridization. A Practical Approach, pp. 73–111. IRL Press, Oxford, U.K.

Barker, J.M., McInnes, J.L., Murphy, P.J. and Symons, R.H. (1985) Dot-blot procedure with [^{32}P] DNA probes for the sensitive detection of avocado sunblotch and other viroids in plants. J. Virol. Methods 10, 87–98.

Diener, T.O. (1987) Introduction. In: T.O. Diener (Ed.), The Viroids, pp. 1–5. Plenum Press, New York.

Forster, A.C., McInnes, J.L., Skingle, D.C. and Symons, R.H. (1985) Nonradioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acids Res. 13, 745–761.

Habili, N., McInnes, J.L. and Symons, R.H. (1987) Nonradioactive, photobiotin-labelled DNA probes for the routine diagnosis of barley yellow dwarf virus. J. Virol. Methods 16, 225–237.

Horst, R.K. and Kawamato, S.O. (1980) Use of polyacrylamide gel electrophoresis for detection of chrysanthemum stunt viroid in infected tissues. Plant Dis. 64, 186–188.

Hutchins, C.J., Keese, P., Visvader, J.E., Rathjen, P.D., McInnes, J.L. and Symons, R.H. (1985) Comparison of multimeric plus and minus forms of viroids and virusoids. Plant Mol. Biol. 4, 293–304.

Imperial, J.S., Rodriguez, M.J.B. and Randles, J.W. (1981) Variation in the viroid-like RNA associated with cadang-cadang disease: evidence for an increase in molecular weight with disease progress. J. Gen. Virol. 56, 77–85.

Keese, P. and Symons, R.H. (1987) The structure of viroids and virusoids. In: J.S. Semancik (Ed.), Viroids and Viroid-like Pathogens, pp. 1–47. CRC Press, Boca Raton, Florida, U.S.A.

Koltunow, A.M., Krake, L.R. and Rezaian, M.A. (1988) Hop stunt viroid in Australian grapevine cultivars: potential for hop infection. Aust. Plant Pathol. 17, 7–10.

Lakshman, D.K., Hiruki, C., Wu, X.N. and Leung, W.C. (1986) Use of [^{32}P] RNA probes for the dot-hybridization detection of potato spindle tuber viroid. J. Virol. Methods 14, 309–319.

Laulhere, J.-P. and Rozier, C. (1976) One-step extraction of plant nucleic acids. Plant Sci. Lett. 6, 237–242.

Leary, J.J., Brigati, D.J. and Ward, D.C. (1983) Rapid and sensitive colorimetric method for visualizing biotin-labelled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc. Natl. Acad. Sci. U.S.A. 80, 4045–4049.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

McInnes, J.L. and Symons, R.H. (1989) Nucleic acid probes in the diagnosis of plant viruses and viroids. In: R.H. Symons (Ed.), Nucleic Acid Probes. CRC Press, Boca Raton, Florida, U.S.A. In press.

McInnes, J.L., Forster, A.C. and Symons, R.H. (1988a) Photobiotin-labelled DNA and RNA hybridization probes. In: J.M. Walker (Ed.), Methods in Molecular Biology. Vol. 4, Humana Press Inc., Clifton, N.J., U.S.A. 401–409.

McInnes, J.L., Forster, A.C., Skingle, D.C. and Symons, R.H. (1988b) Chemical biotinylation of nucleic acid probes with photobiotin. Methods Enzymol. In press.

Meinkoth, J. and Wahl, G. (1984) Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. 138, 267–284.

Newbury, H.J. and Possingham, J.V. (1977) Factors affecting the extraction of intact ribonucleic acid from plant tissues containing interfering phenolic compounds. Plant Physiol. 60, 543–547.

Owens, R.A. and Diener, T.O. (1984) Spot hybridization for detection of viroids and viruses. Methods Virol. V11, 173–187.

Palukaitis, P., Rakowski, A.G., Alexander, D.McE. and Symons, R.H. (1981) Rapid indexing of the sunblotch disease of avocados using a complementary DNA probe to avocado sunblotch viroid. Ann. Appl. Biol. 98, 439–449.

Rezaian, M.A., Koltunow, A.M. and Krake, L.R. (1988) Isolation of three viroids and a circular RNA from grapevines. J. Gen. Virol. 69, 413–422.

Schumacher, J., Randles, J.W. and Riesner, D. (1983) A two-dimensional electrophoretic technique for the detection of circular viroids and virusoids. Anal. Biochem. 135, 288–295.

Smith, F.D. and Banttari, E.E. (1987) Dot-ELISA on nitrocellulose membranes for detection of potato leafroll virus. Plant Dis. 71, 795–799.

Symons, R.H. (1984) Diagnostic approaches for the rapid and specific detection of plant viruses and viroids. In: T. Kosuge and E.W. Nester (Eds.), Plant-Microbe Interactions, Molecular and Genetic Perspectives. Vol. 1, pp. 93–124. MacMillan, New York.

Thomas, P.S. (1983) Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. 100, 255–266.

Van Wezenbeek, P., Vos, P., Van Boom, J. and Van Kammen, A. (1982) Molecular cloning and characterization of a complete DNA copy of potato spindle tuber viroid RNA. Nucleic Acids Res. 10, 7947–7957.

White, B.A. and Bancroft, F.C. (1982) Cytoplasmic dot hybridization: Simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. 257, 8569–8572.