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RECENT EXPERIENCE WITH THE USE OF SYNTHETIC DNA PROBES FOR THE DETECTION OF AVOCADO SUNBLOTCH VIROID

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SUMMARY

Recent experience using 23 oligonucleotides to diagnose avocado sunblotch viroid (ASBV) is described. In a number of field collected samples with sunblotch symptoms, the probe was unsuccessful in detecting the viroid. The sunblotch viroid varies greatly from branch to branch in a tree. Tree to tree variation is also common. However, the probe can be used with a reasonable measure of success to detect sunblotch infected avocado trees.

OPSOMMING

Onlangse proefnemings met 23 oligonukleotiedes om die avokadosonvlekviroid te diagnoseer word beskryf. 'n Aantal avokadoplantmonsters wat in die veld versamel is en wat simptome van sonvlek vertoon het, het negatiewe resultate met die bereide peiler getoon. Die sonvlekviroid wissel egter grootliks in verskillende dele van 'n besmette plant en die viroidkonsentrasie varieër ook grootliks van plant tot plant. Nogtans is gevind dat die peiler met 'n redelike mate van betroubaarheid aangewend kan word om sonvlek-besmette plante op te spoor.

INTRODUCTION

Traditionally, the detection of viroid infected plants has relied mainly on symptom expression in them or in test plants, and assays for the presence of biochemical components associated with disease.

Symptom expression of ASBV may require a large number of indicator plants and long incubation periods. An obvious way to diagnose these agents is specific hybridisation of the infectious nucleic acid with complementary DNA (cDNA) prepared either primed randomly using oligo dT or with a specific sequence primer for reverse transcription. Depending on hybridisation conditions and the source of the sequence used as probe, nucleic acid hybridisation tests are sensitive and specific. The cDNA probes can be amplified ¡n recombinant DNA plasmids, so that repeated isolation of virus for cDNA production with reverse transcriptase will not be needed. Recent developments in synthesis of oligonucleotides (Beaucage & Caruthers, 1981; Matteuccy & Caruthers, 1981) permit the construction of substantial quantities of cDNA to previously sequenced

molecules. In this article we describe our recent experience of using a 23 base oligonucleotide to diagnose avocado sunblotch viroid (ASBV). A detailed review article describing the methodology for preparing synthetic DNA probes and their application for ASBV and tobacco mosaic virus diagnosis has been published recently (Bar-Joseph *et al,* 1986 Ann. Appl. Biol. in press.)

MATERIALS AND METHODS

^{32}P labelling of oligonucleotides. For ^{32}P labelling, 10 µl of a 0,10 µg/ml oligonucleotide solution, 2,2 µl of 10 times concentrated reaction buffer (50 mM tris-hydrochloric acid pH 7,5, 10 mM magnesium chloride and 10 mM 5-dithiothreitol), 1,5 µl (6 units) of T-4-polynucleotide kinase (Biolabs) and 8 µl Adenosine 5' [∂ -^{32}P] triphosphate, 80 uc., 3000 C_i/mM (PB 10168, Amersham Int.) were incubated at 37°C for 30 min. The reaction was terminated by heating to 70°C for 5 min and the mixture of free and labelled nucleotides loaded onto a small SephadexG-50 column and eluted with 5 ml distilled water in 0,5 ml fractions.

Sap extracts. The method followed was that of Owens & Diener, (1981). 5-10 discs, 1,2 cm in diameter (c. 0,15 0,3g) cut from leaves of healthy or infected plants were ground in a pestle and mortar in the presence of sand and 1 1,5 ml of extraction buffer (200 mM K_2HPO_4, 10 mM sodium dithiocarbamate and 0,1% Triton X 100). The extracts were centrifuged for 2 min and the supernatants were heated at 100°C for 3 min prior to the spotting of samples (3 µl) on nitrocelullose filters.

Nucleic acid extracts. The protocol for simple routine ASBV extraction included the following steps:

1) Avocado leaf strips (2 g) were homogenised in 10 ml of 4% paramino salycilic acid (PASA) (Sigma) dissolved in 0,05 M tris-hydrochloric acid, 0,1 M sodium chloride and 0,001 M EDTA, pH 7,0 (STE).

2) The homogenate was transferred to a tube containing 10 ml of STE saturated phenol and 0,1 % 8 hydroxvauinoline sulphate. (Steps 1 and 2 were separated because of safety considerations when processing large numbers of samples).

3) Samples were centrifuged for 10 min at 8000 rpm in a Sorval SS 34 rotor.

4) The upper phase was collected and mixed with 1/9 volume of 3 M Sodium acetate pH 5,5 and 2 volumes of ethyl alcohol. The solution was kept for 2 h at 70°C or overnight at -20°C.

5) Samples were centrifuged as in (3) for 10 min and resuspended in 0,4 ml distilled water. This suspension was designated crude nucleic acid (NA) extract.

6) Samples (3-5 µl) of crude NA extract were spotted on nitrocellulose filters. The filters were prepared by wetting them in distilled water then in 20 X SSC.

7) For further purification the crude NA extract was mixed with 0,9 ml ethyl alcohol and

4,0 ml distilled water and passed through a small column (Isolab) containing 1,5 ml of CF11 cellulose (Whatman) powder suspended in STE and 17% ethanol. The eluate was collected and RNA precipitated as in (4).

8) The pellets were resuspended ¡n a minimal volume of STE. These suspensions were designated partially purified viroid preparation.

In addition we tested two alternative viroid extraction methods which were essentially similar to those of Jordan, Heick, Dodds & Ohr, (1983). They involved modifying the above procedure as follows:

a. Homogenisation of 2-3 g leaf strips with 10 ml 2 STE, 1% SDS and 10 ml STE saturated phenol or 10 ml 1 % PASA and STE saturated phenol.

b. After centrifugation, the upper phase was collected, ethyl alcohol added to 16,5% and suspension transferred to CF 11 column, the eluate collected and processed as in 4, 5 and 6.

Hybridisation with labelled cDNA.

Nitrocellulose filters were incubated for 2 h at 42°C in a prehybridisation solution consisting of 50%formamide, 0,05 M sodium phosphate buffer at pH 6,5, 1 SSC and 0,02% polyvinilpyrrolidone, 0,02% Ficoll 400 and 0,02% bovine serum albumin (Denhardt solution).The filters were removed, drained and incubated for 2-16 h at 37°C in a hybridisation solution containing 50% formamide, 4 fractions each containing 0,5 ml of labelled oligonucleotides, 1 x SSC, 1 x Denhardt solution and 0,05 M phosphate buffer pH 6,5. These hybridisation conditions were chosen as they were found to give effective hybridisation with known infected samples and low background hybridisation with the control healthy samples.

After removing the filters from the hybridisation solution, the filters were washed 4 times for 15 min at room temperature in 500 ml solution containing 2 x SSC, 0,1 % SDS and 0,1% pyrophosphate (NaPP) and 2 times for 15 min at 50°C in 250 ml solution containing 0,05 ml SSC, 0,1 ml 10% SDS and 0,1 ml of 10% NaPP. The nitrocellulose filters were then dried, placed in a photographic plate holder containing an intensifying screen for 2-48 h at room temperature or at 70°C depending on RNA concentrations placed on the filter or on the probe efficiency. In routine screening practice the hybridisation solution containing an effective probe can be repeatedly used for different filters as many as 6-8 times without affecting its diagnostic quality.

DETECTION OF AVOCADO SUNBLOTCH VIROID

Avocado sunblotch is a serious disease affecting the yield and fruit quality of avocado *(Persea americana).* A low mol. wt. circular RNA has been associated with the disease (Thomas & Mohammed, 1979; Dale & Allen, 1979; Palukaitis, Hatta, Alexander, Symons, *et al.* 1979; Semancik & Desjardins, 1980) and infectivity studies with highly purified RNA preparations have recently confirmed the viroid nature of ASBV (Allen, Palukaitis & Symons, 1981; Untermohlen, Drake, Desjardins & Semancik, 1981). The sequence of 247 nucleotide residues of the single-stranded circular RNA from ASBV

was determined by Symons (1981).

We recently examined the hybridisation procedure with two short 20 and 17 nucleotide, DNA probes and found them sensitive and specific for ASBV diagnosis (Bar-Joseph, Segev, Twizer & Rosner, 1985).

The work described in this paper was done mainly with a 23 base probe (CACTCTCTTCCTCCTCAGCACCA) complementary to ASBV RNA sequences 90-112. This sequence was selected as a target for synthesis because of its low homology with other sequences of the circular ASBV molecule (Symons, 1981) and relatively high G and C content (13:23). The probe was found to be highly specific and only the ASBV infected avocado leaf samples gave strong hybridisation spots. A simple PASA phenol extraction method followed by ethanol precipitation was found to give satisfactory ASBV detection even at a sample dilution of 1:1000, although when filters were spotted with unclarified extracts, some non-specific spotting of the healthy control extracts was experienced. These non-specific spots were limited in diameter to the size of the brown spot residing on the filter membrane and seemed to result from a nonspecific trapping of low molecular probe to the crude avocado extract.

All 24 ASBV 'Hass' seedlings which had been inoculated for >2 y gave positive hybridisation with probe 90-112. Most of the isolates tested caused severe leaf and twig symptoms, but similar efficient hybridisation was also obtained with ASBV isolates causing symptomless infection and with one ASBV isolate with a slightly faster mobility in acrylamide gels (Spiegel, Alper & Allen, 1984). However, when we applied the dot-blot hybridisation test on 20 ASBV infected mature trees at three different avocado groves, only 5/6 infected trees were identified in one of the groves sampled, and only 5/8 and 2/6 of the samples collected from infected trees at two other locations (Barkai and Pardesia respectively) gave positive results.

Repeated indexing at monthly intervals of 7 infected trees at Barkai during May, June and July revealed only one positive hybridisation among the 2 non-reacting trees. Table 1 summarises results of multiple sampling of 7 symptom expressing trees at Barkai. The results clearly indicate that ASBV is variably distributed in mature avocado trees. Such variable detection rates among trees showing ASBV symptoms might have been caused either because of low viroid concentrations in some of the infected trees or as the result of the association of ASBV-like symptom with viroid genomes not possessing this particular sequence. Experiments using biological indexing combined with hybridisation tests are continuing in order to establish practical methodology for routine diagnosis of field collected samples.

PROSPECTS, LIMITATIONS AND FUTURE DEVELOPMENTS

A number of field collected samples with ASBV symptoms were not detected or only detected following repeated testing when using ASBV 90-112 probe or a previously prepared ASBV 68-87 probe. Previous studies have shown 1000-10,000 fold variation in ASBV in different trees and branches (Dale & Allen, 1979; Palukaitis *et al,* 1979; Bar-Joseph *et al,* 1985). Analysis of viroid distribution in 7 different closely-spaced planted mature trees further indicated tree to tree variation in the concentration of hybridisable

ASBV molecules in symptom expressing trees. The reasons for this unusual variation are not known and further studies of ASBV multiplication and spread will be needed in order to improve routine ASBV diagnosis. An alternative possibility of involvement of a second viroid pathogen in ASBV like symptomatology should also be considered. Although the results of indexing of field samples clearly indicate that this probe is not effective for detecting all ASBV infected trees, nevertheless the availability of a method for rapid screening enables the elimination of many of the infected budwood source trees within a short period (few months). This warrants the immediate introduction of this method for practical purposes.

TABLE 1

Hybridisation of different leaf samples from seven trees showing ASBV symptoms with a synthetic oligonucleotide probe.

TREE No.	BULK SAMPLING HYBRIDISATION INTENSITY	INDIVIDUAL BRANCH HYBRIDISATION		HYBRIDISATION RATE
		+	+++	
1	+	2	0	2/8
2	+	0	2	2/6
3	+++	2	3	5/10
4	-	0	0	0/6
5	++	0	6	6/6
6	-	1	0	1/6
7	+++	0	7	7/7

Bulk sampling: collected from ten different leaves.

Individual branch sampling: each collected from a main tree branch.

Hybridisation intensity: - negative; +weak; ++intermediate; +++strong.

Hybridisation rate: numerator = number of branches showing hybridisation;
denominator = number of branches tested per tree.

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