

■ Development of a nucleic acid based membrane hybridization test for the detection of ASBVd

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As an addition to its wide portfolio of viroid tests, Agdia has developed a nucleic acid hybridization RNA dot blot test to detect avocado sun-blotch viroid (ASBVd) in leaves of avocado trees. The test is specific to ASBVd and does not cross-react with pospiviroids or other members of the Asunviridae family. The test has a sensitivity of at least 6 pg of ASBVd RNA, which translates to 7 x 10⁷ genome copies. The extraction methodology is a simple one, which utilizes Ames Buffer. The extract is applied directly to the membrane without need for further purification. Sampling methodologies will be discussed.

Como una adición a su portafolio de pruebas de viroides, Agdia Inc. ha desarrollado una prueba de hibridación de ácidos nucleicos en muestras de hojas de árboles de palto impregnadas en membranas para detectar al viroide del manchado solar del palto (ASBVd). La prueba es específica para ASBVd y no reacciona en forma cruzada con miembros de los Pospiviroides ni con otros miembros de la Fam. Asunviridae. La prueba tiene una sensibilidad de al menos 6 pg de ARN de ASBVd lo que se traduce en 7 x 10⁷ copias del genome del viroide. La metodología de extracción de los ácidos nucleicos es simple utilizando el buffer AMES. El extracto aplicado directamente a la membrana no requiere de mayor purificación. Las metodologías para muestreos serán discutidas.

Keywords: Avocado sun-blotch, Asunviridae, Viroid, RNA dot blot

INTRODUCTION

ASBVd is found in avocado growing regions worldwide, such as Australia, Africa, South and North America. Trees infected with ASBVd can result in a loss of yield and / or production of unmarketable fruit.

Symptoms may vary depending on the variant of ASBVd causing the infection (Semancik & Szychowski, 1994). The site of viroid replication has been identified as the chloroplast (Navarro *et al* 1999) and viroid has been isolated from symptomatic and asymptomatic plants (Dale *et al* 1982). Since symptoms are not always present, infected trees are not always identified in a timely fashion, which can allow for spread via pollen, infected budwood or seeds. This makes pro-active testing a critical disease control measure.

MATERIALS AND METHODS

Probe

The probe pBdASBVd [A28], was obtained from Dr. Ricardo Flores. It comprises a dual promoter plasmid, pBluescript II KS+/BamHI, containing a dimer of the ASBVd sequence cloned into the BamHI site. Positive strand (positive control) RNA is produced by *in vitro* transcription using T3 polymerase and negative strand (probe) RNA is similarly produced using T7 polymerase. The probe is expected to generate products which will hybridize to known variants of AVSbD. Isolation, purification, and restriction endonuclease digestion of plasmid DNA was performed according to standard protocols.

In vitro transcription of digested plasmid

T7 RNA polymerase was purchased from Promega. *In vitro* transcription of positive control RNA was done according to the manufacturer's suggested protocols. Production of labeled, negative strand RNA for use as a probe was performed using the Roche DIG RNA Labeling Mix (Cat # 11277073910) and according to the manufacturer's specifications. A final ethanol precipitation was included after transcription in all cases. The concentration of positive control RNA was determined by UV spectrophotometry and copy number was calculated.

Samples and sample extraction

Leaf samples taken from uninfected and infected avocado trees were kindly provided by Dr. Ricardo Flores and were stored at -80°C until needed. Extraction of leaf samples was done according to the Ames and Chloroform Sample Extraction method described by Podlecki *et al* (1993).

Dot blot hybridization and detection

RNA was spotted (2µl) onto nylon membrane. Hybridization and detection was done essentially as described in Podlecki *et al* (1993) with the following modifications in components. The hybridization medium was DIG Easy Hyb (Roche Cat# 11603558001). The detection portion utilizes Blocking Reagent (Roche Cat# 11096176001), Anti-digoxigenin-AP Fab fragments (Roche Cat# 11093274910), and CSPD (Roche Cat# 11655884001). All were used according to the manufacturer's specifications.

Results and discussion

The sequence and abbreviated map of the probe plasmid along with putative transcriptional self-cleavage products are depicted in Figure 1 below. The probe is expected to generate products which will hybridize to known variants of AVSBd. This clone was utilized to produce both the positive control RNA and the probe used in the experiments below.

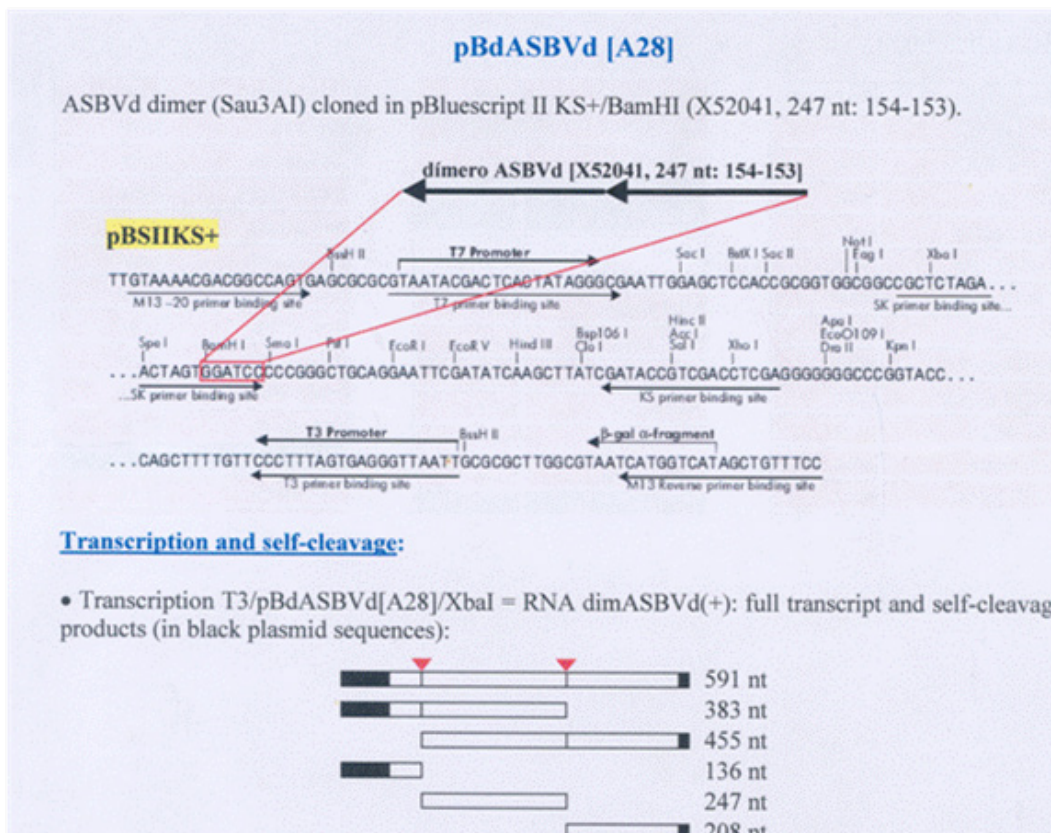


Figure 1: Abbreviated map of the ASBVd probe plasmid with potential cleavage products

The transcribed product is expected to provide probes of varying lengths, capable of binding to immobilized ASBVd RNA. Figure 2 below, portrays a lineup of major known variants of ASBVd (Semancik & Szychowski, 1994). Homologies to the probe sequence range from 96% to 100% identity. It is expected that the probe will therefore easily detect the major variants under the conditions used.

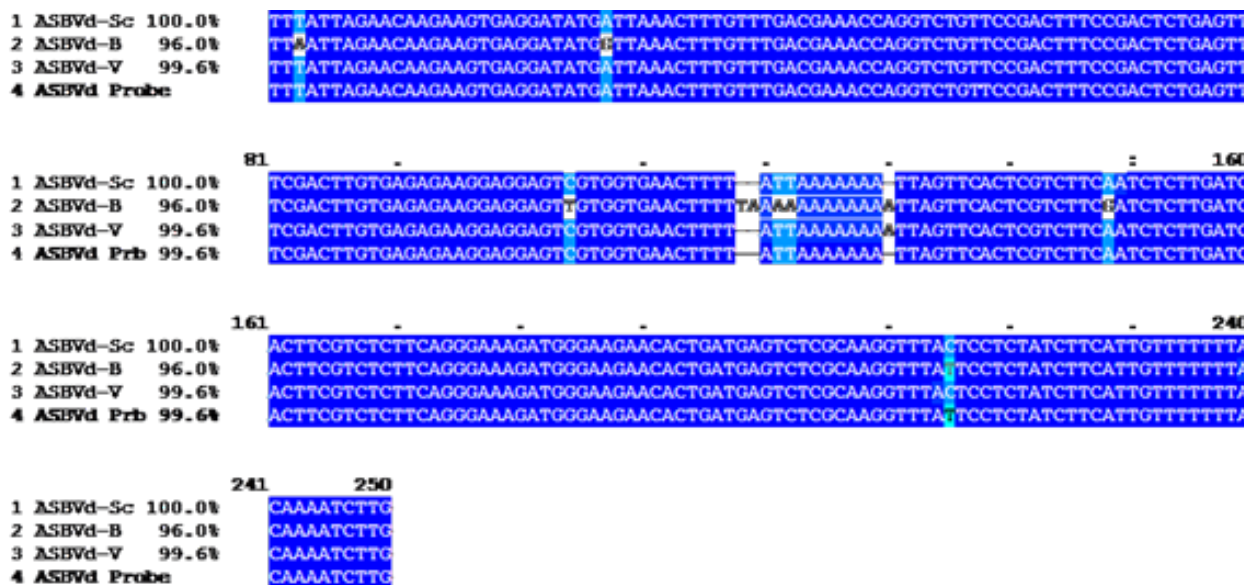


Figure 2: Comparison of the sequences of the major known variants of ASBVd with the probe sequence

Initial efforts focused on the sensitivity and specificity of the assay. To that order, membranes were spotted with purified control RNA of various cloned viroids or extracts from viroid infected tissue. As well, positive ASBVd control RNA was serially diluted, spotted on the membrane, and the membrane was then hybridized against the labeled ASBVd probe (Figure 3). The probe does not cross-react with PSTVd, TCDVd, TASVd, TPMVd, CChMVd, PLMVd, or CSVd. It is also capable of detecting ASBVd RNA down to at least 12.5 pg. Later experiments (not shown) demonstrated a sensitivity of at least 6 pg.

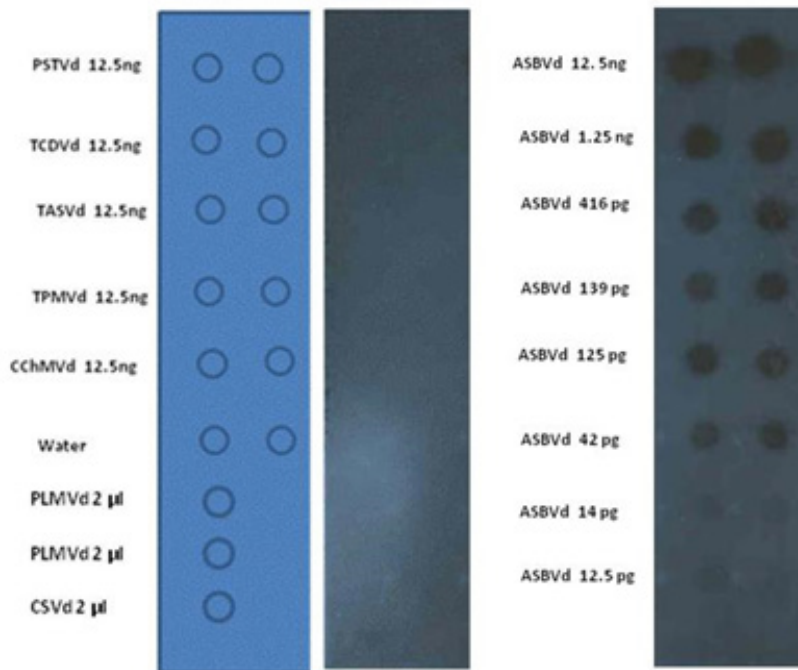


Figure 3: Cross-reactivity and sensitivity

Desiring to have the simplest methods of extraction possible, water and Ames/chloroform were investigated. Both were capable of extracting the viroid from avocado leaves and produced good results. There were no background problems with uninfected tissue and sensitivity initially appeared good. However water extracts showed a tendency to lose titer, even at -80°C. Ames/chloroform extracts demonstrated good stability at -20°C so it was decided to concentrate on that method.

A series of compositing experiments were designed to determine at what ratios of infected to uninfected tissue ASBVd could still be detected. Figure 4 is an example of a blind compositing experiment. Weight to weight composited leaf samples were prepared, in duplicate, by one individual. The samples were transferred to Agdia's Testing and Research departments, where they were extracted by scientists in those departments and membranes were spotted and processed. Composited ratios as high as 1:31 were easily detected by the assay.

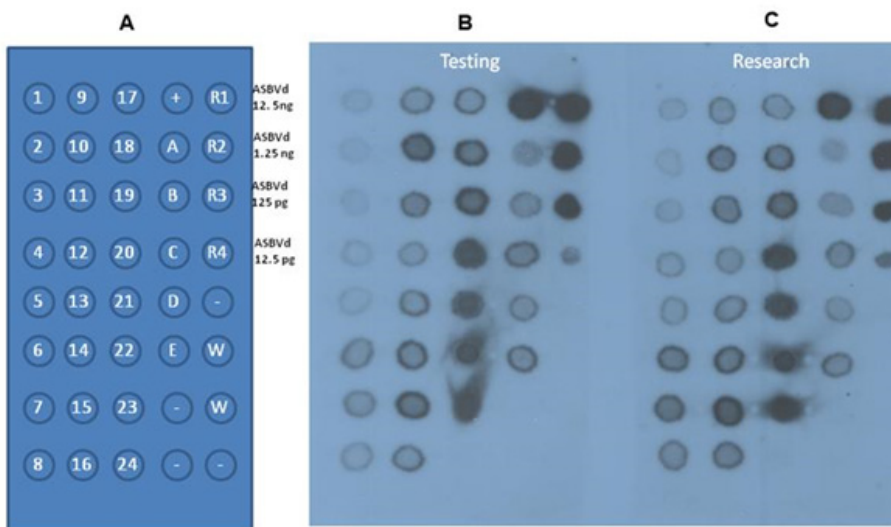


Figure 4: Detection of blind avocado leaf samples. Composited infected:healthy ratios are samples 1-6 (1:31), samples 7-12 (1:15), samples 13-15 (1:7), samples 16-18 (1:3), samples 19-21 (1:1), samples 22-23 (neat +), sample 24 (neat -). Samples A-E (known composites 1:1 through 1:31), R1-R4 (ASBVd RNA dilution series), W (water)

Further exploration of compositing was done with field samples provided by Dr. Luis Salazar. Samples were collected, extracted, and then composited at a ratio of 1:1 or 1:2 infected: uninfected extract. The results, shown in Table 1, demonstrate that, in field samples having varying titers, compositing may not be the best strategy if one is to detect low levels of ASBVd infection.

Table 1: Detection of ASBVd in composited samples from avocado groves

Sample	Volume : volume compositing		
	Neat	1:1	1:2
1	-	-	-
13	+	+	+
14	+	+	+
15	+	+	±
45	+	+	-
52	+	+	+
61	+	+	±
63	+	+	-
64	+	+	±
65	+	+	+
66	+	+	+
83	+	+	+

Given the uneven distribution of ASBVd throughout any given tree, a survey was undertaken, again with the help of Dr. Luis Salazar, to attempt to determine how best to sample trees in an avocado grove. Leaves were sampled from four locations along two different branches of multiple trees. The samples were individually tested for ASBVd. The results of this survey, shown in Table 2, suggest that multiple leaves should be sampled from more than one location on a tree.

Table 2: Sampling strategy for field trees

Sample leaf position	Tree and Branch					
	P04		P13		P16	
	A	B	A	B	A	B
1	+	-	±	+	+	±
2	-	-	+	+	+	-
3	-	-	+	+	+	-
4	-	-	-	+	+	+

CONCLUSIONS

A sensitive and specific nucleic acid hybridization test has been developed for the detection of ASBVd. The assay is capable of detecting as little as 6 pg of ASBVd RNA in a dot blot format. The assay detects infection in leaf samples from both symptomatic and asymptomatic trees. While sample compositing of highly infected samples is well within the scope of the test, similar levels of detection cannot be guaranteed with lower infection rates. Therefore the general recommendation is not to composite samples and to sample multiple areas on a single tree. To date, the assay has been validated only in leaves, not in fruit or flowers.

ACKNOWLEDGEMENTS

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