Avocado sunblotch disease: a persistent viroid infection in which variants are associated with differential symptoms

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Introduction

The avocado sunblotch viroid (ASBVd) has been described as a rod-like RNA structure of 247 nucleotides (Symons, 1981) which induces the sunblotch disease syndrome (Dale et al., 1982; Desjardins, 1987). ASBVd has been regarded as an atypical viroid because of its nucleotide sequence and predicted structure. The A-U-rich base composition and low sequence homology with other viroids mean that ASBVd does not fit the consensus model of structural domains described by Keese & Symons (1985). The property of self-cleavage (Hutchins et al., 1986), recently demonstrated for only one other viroid (the peach latent mosaic viroid; Hernandez & Flores, 1992) is the most striking feature of ASBVd.

The exceedingly high titres of ASBVd, which at times approach the concentrations of host 5S RNA, are also unusual among viroids. The very narrow host range, particularly the species Persea americana Mill., suggests a highly specific relationship between the viroid and its host plant.

The existence of ASBVd variants has been inferred from differences in the nucleotide sequence and molecular size estimates of ASBVd isolates (Palukaitis et al., 1981; Spiegel et al., 1984; Pallas et al., 1988; Rakowski & Symons, 1989). However these proposed strains have never been positively characterized as distinct variants by differences in their biological properties.

This study reports the nucleotide sequences of variants of ASBVd derived from tissues exhibiting different symptoms. A progression in disease development associated with sequence variants of ASBVd is also presented.

Methods

Plant culture and bioassay. Healthy and infected avocado, P. americana cv. Hass, were grown under standard greenhouse conditions as seedlings or as clonal propagations grafted onto cv. Topa Topa or cv. Zutano seedlings from registered source trees tested as negative for all known avocado diseases. Plants were infected by graft-inoculating either rootstock or scion with tissue pieces from infected trees. Bioassays for sunblotch disease were accomplished by inoculation of cv. Hass clonal propagations and monitoring for the appearance of stem streaks or foliar symptoms for at least 2 years.

Viroid culture and purification. Apical tissues or symptomatic leaves of avocado were used for nucleic acid extraction. Fresh tissue was either used immediately or pulverized in liquid nitrogen and stored at −20 °C for processing later. ASBVd was recovered from these tissues by the CF-11 cellulose trapping protocol described for the purification of grapevine viroids (Szychowski et al., 1988). ASBVd was present in the aqueous phase after sodium sulphite-phenol extraction.

Viroid detection by sequential (s) PAGE and PCR. Viroid RNA was detected and purified by sPAGE (Semancik & Harper, 1984; Riverabustamante et al., 1986). Denaturing 8 M-urea gels were stained with ethidium bromide for the viroid purification process or silver for greater sensitivity in viroid detection (Igloi, 1983). Purified viroids were obtained by electroelution of the ethidium bromide-stained bands containing the circular viroid molecules using an IBI Model UEA unidirectional electrophoret.

Full-length ASBVd cDNA was generated from partially purified nucleic acid preparations consisting of ethanol concentrates or 2 M-lithium chloride-soluble samples from phenol extracts. ASBVd-specific synthetic oligonucleotide primers complementary to residues 161 to 179 or homologous to residues 180 to 198 of ASBVd SB-1 (Symons, 1981) were used. This primer pair covers an ASBVd site which had shown no sequence heterogeneity in the 51 cDNA clones analysed by Rakowski & Symons (1989).

First-strand cDNA was synthesized from RNA extracts by reverse transcription (Visvader & Symons, 1985). The cDNA was amplified by PCR using Taq polymerase in buffer and 2.5 mM-Mg²⁺. PCR was performed over 30 cycles of 92 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The products were analysed on 2 % agarose gels, and stained with ethidium bromide after electrophoresis.
Fig. 1. A healthy leaf of avocado cv. Hass (a) compared with ASBVd-infected leaves with bleached (b), variegated (c) and symptomless carrier (d) symptoms. Note the close association of the bleached symptoms with petiole and vascular tissues.

Fig. 2. A 5% polyacrylamide gel containing 8 M-urea after sPAGE and staining with silver. Viroid preparations from avocado leaves of healthy trees (lane 1) and symptomless carrier trees (lane 2), a non-symptomatic half-leaf (lane 3) and the symptomatic half (lane 4) of a variegated leaf and the non-symptomatic (lane 5) and symptomatic (lane 6) portions of a bleached leaf.

Nucleotide sequencing. The PCR product from several reactions was pooled and purified by electrophoresis in 2% low melting point agarose. The resulting DNA was treated with the Klenow fragment of DNA polymerase I in the presence of all four dNTPs before being ligated into the dephosphorylated, Smal-digested DNA of the phage M13 mp18. All clones were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1980) using T7 DNA polymerase (Sequenase version 2.0, USB).

Results

Differentiation of foliar symptoms in infected avocado

Avocado sunblotch disease has been characterized by the occurrence of a highly variable syndrome including stem streaks, fruit discoloration and lesions, as well as a variety of different foliar symptoms (Dale et al., 1982; Desjardins, 1987). With continuous observation of infected trees under greenhouse conditions over several years it was possible to identify two distinct and clearly defined patterns of foliar symptoms. These appear as either an intense chlorotic zone associated with vascular tissues or variegation which is spread throughout the leaf (Fig. 1). These patterns can be found on different shoots of the same tree or as isolated cases on different trees. Distinct foliar reactions were divided into three basic types, bleached (B), variegated (V) or symptomless carrier (Sc).

The most common initial symptom of sunblotch
Variants of ASBVd

Fig. 3. A 2% agarose gel stained with ethidium bromide of the PCR products produced from viroid preparations. The preparations and the corresponding lane numbers are the same as those in Fig. 2. The position of 242 and 320 bp markers, above and below the full-length ASBVd product, are indicated. Larger products from viroid-infected leaf extracts correspond to multimeric forms known to occur in ASBVd infection.

Table 1. Segregation in symptom-expression of sunblotch syndrome associated with ASBVd variants

<table>
<thead>
<tr>
<th>Symptom (half-leaf or area)</th>
<th>Viroid analysis (sPAGE and PCR)</th>
<th>Titre</th>
<th>Migration rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleached</td>
<td></td>
<td>++ +</td>
<td>Slow</td>
</tr>
<tr>
<td>non-symptomatic</td>
<td>*</td>
<td>+ *</td>
<td>Slow</td>
</tr>
<tr>
<td>Variegated</td>
<td></td>
<td>++ +</td>
<td>Fast</td>
</tr>
<tr>
<td>non-symptomatic</td>
<td></td>
<td>++ +</td>
<td>Fast</td>
</tr>
<tr>
<td>Symptomless carrier</td>
<td></td>
<td>++ +</td>
<td>Fast</td>
</tr>
</tbody>
</table>

* Positive detection by PCR.

Infection is the appearance of stem streaks. However the first foliar symptoms noted were, almost exclusively, small, highly defined regions that were devoid of colour and that had a bleached appearance. This symptom was never widespread in affected trees and was usually restricted to a single actively growing tip. The few leaves which displayed this symptom sometimes had pronounced bleached petioles and midvein-clearing with adjacent small irregular areas of bleached tissue.

In some cases new growth from shoots containing bleached leaves also developed a variegated leaf pattern that resembled genetic variegation. In other diseased trees, new tissue appeared healthy and symptomless. Among variegated leaves it was possible to find a asymptomatic half-leaf clearly segregated by the midvein from a non-symptomatic half-leaf. It was found to be impossible to differentiate between the symptomless leaves which developed on symptomatic shoots and healthy leaves from trees that were disease-free.

ASBVd associated with tissues from infected avocado

An analysis of the viroid content of different tissue types was carried out. High-titre accumulations of ASBVd were detected by sPAGE. Fig. 2 shows the sPAGE pattern of ASBVd in extracts from single leaf samples of bleached and variegated symptomatic tissues as well as a symptomless carrier source. The viroid was readily detected in both variegated and bleached tissues. ASBVd was also evident in extracts from non-symptomatic tissue from variegated or symptomless carrier sources. Extracts from the symptomless portions of bleached leaves contained only a very weak band, which was insufficient for positive identification of ASBVd (Fig. 2, lane 5).

PCR amplification was employed to increase the sensitivity of detection from tissues that tested negative using sPAGE. Included in these analyses were extracts from the non-symptomatic regions of bleached leaves as well as non-symptomatic tissue from new growth on shoots which had previously displayed symptoms but

Fig. 4. A 5% polyacrylamide gel containing 8 M-urea after sPAGE and staining with ethidium bromide. Single and mixed viroid preparations from infected tissues displaying different symptoms. Lane 1, symptomless carrier and variegated leaf tissue; lane 2, symptomless carrier and bleached tissue; lane 3, bleached tissue alone; lane 4, bleached and variegated tissues.
tested negative for ASBVd using sPAGE. Fig. 3 demonstrates the strong positive reaction that can be obtained from tissues containing extremely low concentrations of ASBVd. All ASBVd-containing tissue could be detected by these procedures.

ASBVd could be detected only by PCR in symptomless leaves found in new growth from shoots containing bleached leaves. However, symptomless leaves that had developed from shoots with variegated leaves usually contained high titres of ASBVd readily detected by sPAGE. A similar relationship between the ASBVd in variegated and symptomless carrier leaves can be proposed in which the symptomless portion of a variegated leaf can be considered to be a type of symptomless carrier tissue.

**ASBVd variants derived from avocado tissue with distinct symptoms**

The inhibition of viroid spread within leaf tissue expressing the bleached symptom, in conjunction with the unrestricted movement in both variegated and symptomless carrier tissues, suggests a possible segregation in the viroid population. These proposed ASBVd variants may have been selected by interaction with the host.

When ASBVd preparations from bleached tissue, together with samples from either variegated or symptomless carrier tissues were separated by sPAGE, a well-defined doublet was evident (Fig. 4, lanes 2 and 4). Thus a discrete ASBVd variant (designated ASBVd-B) could be defined as the predominant viroid species in the bleached tissue. The slower migration rate of ASBVd-B in denaturing gels suggests that it is a larger molecule than ASBVd-V or ASBVd-Sc (Fig. 4).

The sPAGE profiles of mixtures of viroid preparations from variegated and symptomless carrier tissues did not indicate different viroid forms (Fig. 4, lane 1). Therefore a size and/or conformation similarity is implied between the viroid found in variegated (ASBVd-V) and symptomless carrier (ASBVd-Sc) tissues. A summary of these relationships is presented in Table 1.

**Nucleotide sequence of ASBVd variants**

The primers used to generate full-length cDNA clones of the three ASBVd variants were made using the sequences of regions complementary (residues 161 to 179) and homologous (residues 180 to 198) to ASBVd SB-1 (Symons, 1981) in which no nucleotide exchanges have previously been reported (Pallas et al., 1988; Rakowski & Symons, 1989). The three full-length cDNA clones of each ASBVd variant were found to have lengths of between 247 and 250 nucleotides. The larger variants (249 and 250 nucleotides) were found in ASBVd-B preparations whereas both ASBVd-V and ASBVd-Sc clones had lengths of 247 and 248 nucleotides.

The sequences of clones from the three ASBVd variants and their proposed secondary structure, based on that suggested by Symons (1981) for ASBVd SB-1 are shown in Fig. 5. A comparison of the nucleotide changes found in the clones of ASBVd-B, ASBVd-V and ASBVd-Sc indicates that all exchanges involve either A or U residues (Table 2).

Clones of ASBVd-Sc were similar to ASBVd SB-1. No exchanges in the left terminal loop were found in ASBVd-V and, of the three residue exchanges in the right terminal loop, two were unique. A greater number of exchanges were noted in ASBVd-B clones, and these were concentrated in the right terminal loop. The two most active regions, residues 105 to 119 and 120 to 128, had exchanges or additions involving U and A residues respectively.

These changes resulted in the enlargement of a poly(A) right loop in ASBVd-B when compared to the loop structure of ASBVd-Sc and ASBVd-V suggested by the model of Symons (1981). When the FOLD program (Zuker & Stieger, 1981) was employed, which calculates the RNA secondary structure of minimum free energy, the right terminal sequences of all three variants were predicted to be poly(A) loops (Fig. 6). The ASBVd-B loop would be larger (nine bases) than those of ASBVd-Sc (five bases) or ASBVd-V (six bases) using this model. In addition, the overall structures of ASBVd-Sc and ASBVd-V were most similar.

**The progression of symptom development after inoculation with sunblotch-infected tissues**

The segregation of ASBVd variants that occurs in avocado can be targeted by the distinctive symptoms identified here. Since the transmission of sunblotch disease by tissue extracts as well as purified ASBVd is very difficult to achieve and unpredictable (Allen et al., 1981; J. S. Semancik, unpublished results), tissue implants were used. Thus any effects induced by low-concentration species in the ASBVd population, such as ASBVd-B, had a better opportunity for expression.

Evidence of transmission, both by visual identification of symptoms and by sPAGE detection, occurred in 3 to 8 months. In all cases, regardless of the inoculum source, the initial foliar symptom was bleached leaves. From this, it may be inferred that the ASBVd-B variant persists in all ASBVd populations even when masked from sPAGE detection by very high levels of ASBVd-V and ASBVd-Sc. As growth continued in avocado inoculated with the bleached tissue, the bleached symptoms were expressed at irregular intervals.

Plants receiving inoculum from the variegated or
Variants of ASBVd

Fig. 5. Nucleotide sequences of representative clones of the three viroid variants ASBVd-Sc clone 1 (a), ASBVd-V clone 14 (b) and ASBVd-B clone 3 (c).

Table 2. Sequence heterogeneity among ASBVd variants associated with different symptoms

<table>
<thead>
<tr>
<th>Residue position</th>
<th>Nucleotide change*</th>
<th>ASBVd-B</th>
<th>ASBVd-V</th>
<th>ASBVd-Sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left terminal loop</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 U→A†</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>239 A→G</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right terminal loop</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>115→118 +U</td>
<td>+†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>119 A→U</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120→121 UU→AA</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122→128 +A†</td>
<td>+†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122→128 +2A†</td>
<td>+†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122→128 +3A</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other exchanges</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 A→G</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105 C→U</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>109 G→U</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146 A→G</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>213 C→U</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The residue number and nucleotide exchange are as described for ASBVd SB-1 (Symons, 1981).
† Exchanges previously reported by Rakowski & Symons (1989).
‡ Exchange is shared by two or more clones.

Symptomless carrier tissues ultimately developed variegated leaf symptoms. Analysis by sPAGE showed that the ASBVd-V form became the predominant variant in infected trees. This is consistent with the prevalence of variegated symptoms. With persistent infection over a period of years, it was possible to recover symptomless shoots containing ASBVd from stems which still expressed the variegated symptoms. From these shoots, symptomless carrier plants were produced that, to date,
have never expressed symptoms even though high titres of the viroid have been found throughout the foliar tissues.

A schematic outline for the developmental transition from the primary phase of infection, characterized by bleached leaf symptoms, to the emergence of variegated or symptomless carrier tissues is presented in Table 3.

**Table 3. An outline of the developmental transition of symptoms in avocado infected with different tissue samples**

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Symptom development</th>
<th>Additional growth flushes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleached (B)</td>
<td>B -* NS* → B → NS → B → NS</td>
<td>→ B</td>
</tr>
<tr>
<td>Variegated (V) or</td>
<td>V -* V → NS → V</td>
<td>→ V</td>
</tr>
<tr>
<td>Symptomless (Sc)</td>
<td>Sc -* NS → NS → Sc</td>
<td>→ NS</td>
</tr>
</tbody>
</table>

* NS, Non-symptomatic.

Discussion

Mechanisms by which viroids are segregated or unevenly distributed within woody host tissues (Hadas et al., 1989; Duran-Vila et al., 1991) are not well understood. Citrus viroid mixtures which remain stable for years in sweet orange [Citrus sinensis (L.) Osb.] or citron (C. medica L.) often segregate into a number of patterns in grapefruit (C. paradisi Macf.) and mandarin (C. reticulata Blanco). Loss of specific viroids from a complex usually occurs with a mixture of unrelated viroids. Viroid titre and the severity of symptoms do not appear to be key factors in the loss of a component from a mixture.

A contradiction exists in the detection of ASBVd. This viroid, which can accumulate to extremely high and readily detectable titres is, in many cases, impossible to detect in symptomless tissues from trees previously testing positive for sunblotch disease (Bar-Joseph, 1990). This suggests an uneven distribution of ASBVd in host tissues or a localized accumulation of distinct viroid variants. The reduction or exclusion of ASBVd in the non-symptomatic portions of a symptomatic leaf has never been noted (Semancik & Desjardins, 1980).

The molecular size differences between variants was previously noted by PAGE analysis under non-denaturing conditions and linked with the presence or absence of symptoms (Spiegel et al., 1984). This hypothesis is not sustained by these data. A difference in the molecular size of variants can be detected in symptomatic tissues displaying either bleached or variegated symptoms. No size heterogeneity is detected by sPAGE between ASBVd variants from symptomatic (V) or symptomless (Sc) tissue extracts.

When viroid segregation is marked by the appearance of specific symptoms, enrichment of particular sequence variants may occur. From this it becomes possible to select tissues visually which contain distinct sequence variants from a population as shown here with variants ASBVd-B, ASBVd-V and ASBVd-Sc.

Supporting evidence for the segregation of distinct viroid populations within tissues can be drawn from the marked restriction of movement of ASBVd-B from symptomatic to non-symptomatic regions of single leaves displaying bleached symptoms. Since bleaching is the initial foliar symptom of sunblotch disease, a primary role in transmission can be inferred for ASBVd-B. Thus, the low specific infectivity of sunblotch disease preparations might be explained by the low titres of ASBVd-V. The ASBVd-B variant can be detected even in the vast excess of ASBVd-V and ASBVd-Sc that is characteristic of established late infections (Rakowski & Symons, 1989).

The two predominant ASBVd variant populations, ASBVd-V and ASBVd-Sc, are not restricted in host tissues. However these forms of ASBVd are not common during the initial phases of sunblotch infection. They appear as secondary forms which tend to accumulate with persistent infections.

The predominant sites of sequence heterogeneity in ASBVd are the terminal loops (Pallas et al., 1988; Rakowski & Symons, 1989) and the nucleotide differences that were found in the three variants reflect this. However, this is unlike the more conserved sequences in the T1 and T2 domains of other viroids (Keese et al., 1988).

During cataloguing of the sequences of 51 ASBVd cDNA clones (Rakowski & Symons, 1989), one sequence variant (A-1) was found to be represented in 19 of the 23 clones from a single isolate and was identical to ASBVd SB-1 (Symons, 1981). The ASBVd-Sc clones sequenced here were similar to ASBVd SB-1, with one sequence variant, ASBVd-Sc clone 1, identical to ASBVd SB-1.

The high titre isolate of sunblotch disease, ASBVd-Sc, is widely distributed throughout avocado tissues and is therefore probably equivalent to the isolate originally sequenced by Symons (1981).

The A-2 sequence variant of Rakowski & Symons (1989), larger than A-1 by one nucleotide and found in four cDNA clones, was identical to ASBVd-V clone 14 sequenced here. All of the larger variants (between 249 and 251 nucleotides) reported by Rakowski & Symons (1989) were represented by only one or two cDNA clones. These clones were found at the lowest frequency and are similar to ASBVd-B.

The exchanges and additions in the right-hand loop of ASBVd-B would be predicted to lead to a larger open terminal region. The proposed enlarged poly(A) right
loop of ASBVd-B introduces a unique structural modification, with implications for the proposed primary role of this variant in the initiation of sunblotch disease. Goodman et al. (1984) suggested that viroid replication might be initiated by the binding of DNA-dependent RNA polymerase II to a terminal loop of the viroid molecule and this conformational alteration might function as the specific binding site.

The progression of symptoms and viroid production from initiation to development of sunblotch infection displays the characteristics of both acute and persistent forms of infection. The initial phase of sunblotch disease is characterized by the detection of the ASBVd-B variant in a self-limiting reaction that is suggestive of an acute form of infection. The disease expression initiated by ASBVd-B then develops into a persistent latent infection with periodic but infrequent expression of either bleached or variegated symptoms.

Ultimately, sunblotch disease can best be described as a chronic infection with the continuous and measured production of high titres of ASBVd-Sc throughout the host. This final host–pathogen relationship is very difficult to discern visibly since it is not accompanied by any symptoms. However, this phase is characterized by a marked reduction in fruit production and a corresponding increase in seed transmission in some cultivars (Desjardins, 1987).

The high titres of ASBVd-Sc detected in symptomless tissues indicate a transition of the sunblotch disease agent to that of a non-antagonistic RNA which is interfaced with host-regulated nucleic acids. Nevertheless, since extracts from these tissues have the ability to induce the acute or bleached form of infection, ASBVd-B must persist at a minimal level, above that required to cause infection, even in the presence of very high titres of ASBVd-Sc. Alternatively, some process can be postulated for the reactivation of ASBVd-B from the ASBVd-Sc pool by enlargement of the right terminal loop.

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References


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