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Molecular characterization of an almond isolate of hop stunt viroid (HSVd) and conditions for eliminating spurious hybridization in its diagnosis in almond samples*

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Abstract

Hop stunt viroid (HSVd) has a wide range of hosts including herbaceous and woody plants. Recently, HSVd was demonstrated to infect almond trees (Astruc et al., 1996). In this work, we present the molecular characterization of an almond HSVd isolate and report on the problems encountered in the diagnosis of HSVd in almond tissue through dot-blot non-radioactive hybridization procedures. False positives were eliminated through incubation of the membranes with RNase at high ionic strength after hybridization. Further experiments which included Northern hybridization, RT-PCR coupled to Southern hybridization, and cloning and sequencing suggested that spurious hybridization signals were due to host RNAs with sequence similarity to HSVd. Genetic characterization of the almond isolate demonstrated the existence of two new HSVd sequence variants named HSVd.alm1 and HSVd.alm2. The changes of HSVd.alm1 and HSVd.alm2 were located at residues variable among HSVd sequences, in loops on the left part of the rod-like molecule that includes the pathogenic (P) domain. Multiple alignments with all the available HSVd sequences and subsequent phylogenetic analyses revealed that the two new almond sequence variants were included in the previously described *Prunus* group of HSVd sequences.

Almond is one of the most important fruit crops in the Southeast of Spain accounting for 12% of the world production. Almond crops in the Mediterranean basin are affected by several syndromes that in most cases have a proposed viral etiology (Di Terlizzi et al., 1994; Martelli and Savino, 1997). Most of these syndromes have been associated with viruses belonging to the ilarvirus genus and occasionally to the thricovirus apple chlorotic leaf spot virus. Only very recently, has the presence of a viroid been observed in almond (Astruc et al., 1996).

Viroids are the smallest known pathogens of plants. They are single-stranded, circular RNAs with rod-like structure and no capsid protein or detectable messenger activity (for reviews see Diener, 1987; Semancik, 1987; Flores et al., 1997). The replication and spread of viroids rely entirely on interactions between their RNA genome and host components. Hop stunt viroid (HSVd) is the viroid with the broadest host range known so far, and infects hop, cucumber, grapevine, citrus, plum, peach, pear, apricot and almond (Shikata, 1990; Astruc et al., 1996). In some hosts, such as grapevine (Shikata,

*The sequences described in this work have been deposited in the EMBL databank with the accession numbers AJ011813 and AJ011814.

1990; Polivka et al., 1996) and apricot (Astruc et al., 1996), the infection appears to be latent. In the case of apricot, it is worthwhile to note the high incidence observed in the Southeastern parts of Spain, where HSVd is present in 81% of the trees (Cañizares et al., 1998). In other hosts, specific disorders such as hop stunt (Shikata, 1990), dapple fruit of plum and peach (Sano et al., 1989) and citrus cachexia (Diener et al., 1988; Semancik et al., 1988) have been associated with HSVd infection. HSVd isolates were separated into three groups after considering overall sequence homologies. Since isolates within each group had only few isolation hosts, the groups were named plum-type (peach, plum and grapevine isolates), hop-type (hop, grapevine, peach and pear isolates) and citrus-type (citrus and cucumber isolates) (Sano et al., 1989; Shikata, 1990). More recently, Kofalvi et al. (1997) characterized nine new HSVd variants from different *Prunus* sources, and the grouping of variants was redefined to include two new groups. This work also revealed that a number of HSVd isolates probably derived from recombination events and that the previous hop-type group itself is likely to be the result of a recombination between members of the plum-type and citrus-type groups.

The present study was initiated to conduct an extensive survey among almond trees from distinct geographical origins using the previously described non-radioactive hybridization approach for HSVd (Astruc et al., 1996), as well as to characterize all the HSVd almond isolates identified. Leaf samples from cultivars of almond were obtained from a collection kept at Centro de Edafología y Biología Aplicada del Segura (Murcia, Spain). The different samples analyzed were from cultivar 'Titan', 'Non Pareil' and 'Texas' originally from United States, the French cultivar 'Ferragies', and 'Achacosta', 'Garrigues', 'Atocha', 'Peraleja', 'Ramillete', and 'Marcona' from Spain. As positive control, leaves of GF 305 peach (*Prunus persica* L.) inoculated with the HSVd isolate causing the plum dapple fruit disease (Sano et al., 1989) were provided by J.C. Desvignes (CTIFL, Pringonrieux, France). Low molecular weight RNA preparations were obtained by phenolic extraction of samples followed by non-ionic CF-11 cellulose (Whatman, England) chromatography, as previously described (Pallás et al., 1987). For dot-blot hybridization, samples were denatured as described (Astruc et al., 1996), applied to nylon membranes (Boehringer Mannheim, Germany), hybridized to a digoxigenin-labelled RNA HSVd probe (Astruc et al., 1996), and detected as reported (Pallás et al., 1998). Six out of eleven samples analyzed (Figure 1a) showed a clear positive hybridization signal evident even in the 1/10 dilutions, while three additional samples gave weaker signals. The intensity of the signals varied among different samples but all were clearly above the background level of uninfected controls (sample 12, Figure 1a) and some close to the levels of the positive control of HSVd-infected GF-305 peach (sample 13,

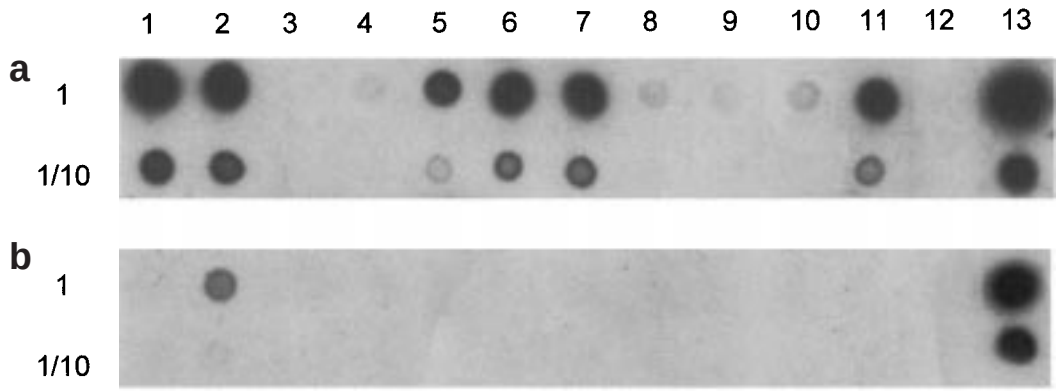

Figure 1. HSVd detection in different almond cultivars by hybridization with a digoxigenin-labelled HSVd-specific RNA probe. Nylon membranes were untreated (a) or RNase A treated after hybridization (b). The cultivars analyzed were 'Titan' (1), 'Non Pareil' (2), 'Peraleja' (3), 'Ferragies' (4), 'Chellastone' (5), 'Texas' (6), 'Achacosta' (7), 'Garrigues' (8), 'Atocha' (9), 'Ramillete' (10) and 'Marcona' (11). The negative control was the apricot 'Ugento' (12) and the positive control was a HSVd infected GF-305 peach (13). Undiluted samples and ten-fold dilutions were dotted onto the membranes. The films were exposed for 20 min.

Figure 1a). Analysis of the samples by gel electrophoresis demonstrated that the concentration of host nucleic acids was similar in all of them (not shown).

From numerous experiments conducted in our laboratory with the above described protocols, it is concluded that positive hybridizations of apricot and peach samples account for HSVd infection, since they are routinely confirmed with Northern analysis and/or RT-PCR (data not shown). However, most of the positive samples shown in Figure 1a failed to show the presence of the viroid in the parallel Northern and RT-PCR analyses (see below). Experiments were designed to evaluate the reliability of the hybridization signals observed in almond samples. RNase A digestion under high ionic strength conditions was used as described previously (Podleckis et al., 1993), in order to reduce non-specific signals; membranes were treated after probe hybridization with RNase A (2 µg/ml) in 2× SSC at 37 °C for 15 min. Surprisingly, the only signals that remained after ribonuclease treatment were those corresponding to the HSVd-infected peach control and to the previously identified 'Non Pareil' almond cultivar (Figure 1b, samples 13 and 2, respectively). The remaining putative positives disappeared following digestion. It has to be noted that the initial signals from cultivars 'Titan' and 'Non Pareil' were similar (Figure 1a, samples 1 and 2); however, after RNase A treatment the putative hybridization corresponding to 'Titan' was completely eliminated, while that from 'Non Pareil' was only reduced albeit still indicative of viroid infection. Most significantly, the hybridization of the positive control (from infected peach) was almost unaffected by RNase A digestion.

It was conceivable that the elimination of the signals from most of the samples after digestion was due to a very low accumulation of HSVd, which would render it undetectable under the new experimental conditions. On the other hand, and given the problems encountered, even the HSVd infection detected in 'Non Pareil' could be questioned. To clarify these issues, additional validation experiments were carried out.

First, Northern blot hybridizations after double polyacrylamide gel electrophoresis (PAGE) (Figure 2a) were conducted for two almond samples that gave positive signals in the initial dot-blot hybridizations ('Titan' and 'Non Pareil', samples 1 and 2, respectively, in Figure 1a) and one sample that gave no signal ('Ferragies', sample 4 in Figure 1a). In the double PAGE protocol the RNA preparations were separated by two consecutive non-denaturing and denaturing electrophoreses through 5% PAGE (Schumacher et al., 1983; Flores et al., 1985), electrotransferred to nylon membranes, and hybridized and detected as described above. Hybridization bands of the electrophoretic mobility of circular and linear HSVd were detected only in the positive control and in 'Non Pareil' (lanes 4 and 1), unequivocally demonstrating HSVd infection, but not in 'Titan'. Secondly, the same RNAs

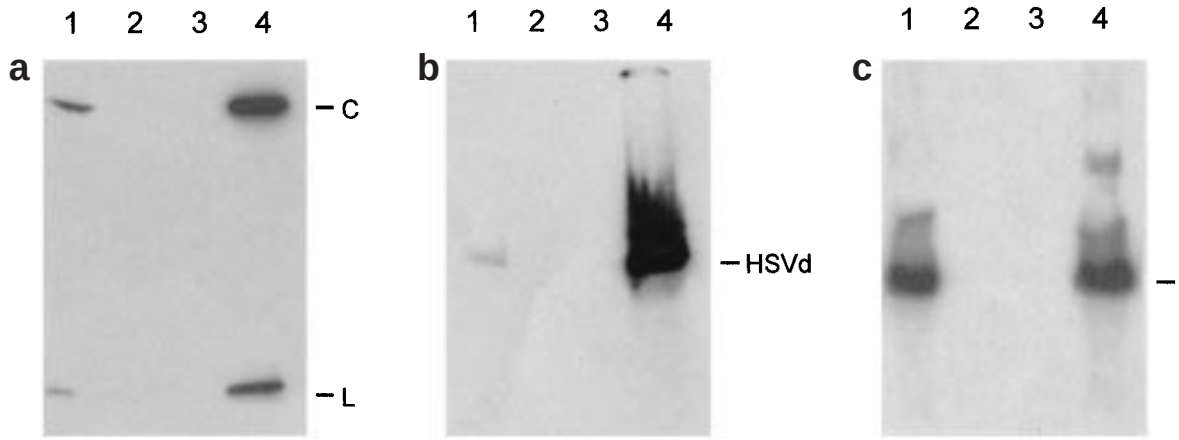

Figure 2. Northern blot hybridization analysis of HSVd after separating total nucleic acids samples from almond leaves by double PAGE (5%) (a) and non-denaturing 5% PAGE (b) conditions, and Southern hybridization of RT-PCR products obtained with HSVd specific primers (c). The samples were almond cultivars 'Non Pareil', 'Titan' and 'Ferragies' (lanes 1, 2 and 3, respectively) and HSVd-infected peach GF-305 (lane 4). In (a), both circular and linear forms of the HSVd can be observed and are marked. In (c), the full-length amplified product is marked. Membranes were hybridized to a digoxigenin-labelled HSVd riboprobe. Films were exposed for 30–60 min.

were also separated in a non-denaturing 5% PAGE and again subjected to Northern hybridization (Figure 2b). This experiment was conducted to rule out the possibility that HSVd RNAs could bind to other cellular components and be lost in the double PAGE analysis (in which only a portion of the first native gel is cut and applied on top of the second denaturing one). The results of the two Northern analyses were coincident (compare Figure 2a and b). Finally, a confirmation of these results was made through Southern blot hybridization of RT-PCR amplified cDNA fragments (Figure 2c). The products from PCR amplification with the HSVd-specific primers (see below) were separated by non-denaturing 5% PAGE, blotted, and hybridized. Again, a positive signal was detected only in the case of the positive control and in 'Non Pareil'.

In conclusion, although some samples of almond showed a remarkable strong dot-blot hybridization signal, our data demonstrated that in the case of almond samples there are difficulties with this diagnosis procedure that require the application of stringent treatments after hybridization. It must be stressed that this is not the case for other *Prunus* hosts (e.g., apricot and peach), in which similar or even lower level of dot-blot hybridization (with no RNase treatment) is indicative of viroid infection (Astruc et al., 1996, data not shown). In line with these findings, the previously reported HSVd infection of pomegranate (Astruc et al., 1996) in which Spanish pomegranate samples gave a clear positive signal must be re-evaluated with the more stringent post-hybridization conditions used in the present work.

The results described above can be explained by considering the presence of a host component that hybridizes even at high stringency conditions and causes the undesired results. We would like to mention that, in the course of our research, DNAs amplified by PCR with HSVd-specific primers were recovered from HSVd-free almond samples. The size of these amplification products was close to that of HSVd, which initially confounded our conclusions (as might do in certification programs), and made necessary the use of a Southern approach (Figure 2c). Upon cloning and subsequent sequencing, it became evident that some of those amplicons corresponded to almond ribosomal RNAs that had significant sequence similarity (up to 57%) with some portions of the HSVd sequence (data not shown). Our conclusion is that, in the absence of the HSVd in the extracts, almond host RNAs can hybridize to the HSVd riboprobe giving a spurious hybridization. Viroids have been shown to exhibit significant sequence similarity to cellular RNAs such as small nuclear RNAs (Solymosy and Kiss, 1985), group I introns (Dinter-Gottlieb, 1986), 7SRNA (Haas et al., 1988) and ribosomal RNAs (Meduski and Velten, 1990; Jakab et al., 1986). Several of the proposed mechanisms of viroid pathogenicity are suggested by sequence similarity between either plus or minus strand viroid and these RNA components.

Using a proof-reading thermostable DNA polymerase, HSVd-specific RT-PCR was applied to 'Non Pareil' samples to isolate and characterize the corresponding HSVd sequences. RT-PCR, cloning of PCR products and sequencing of the cloned inserts were performed as described (Astruc et al., 1996; Kofalvi et al., 1997). The oligonucleotides used were the antisense 26-mer VP-19, complementary to HSVd residues 60–85, and the sense 27-mer VP-20, which contains residues 78–102. After PCR amplification and subsequent cloning, three independent full-length cDNA clones with distinct sequences were characterized, demonstrating the existence of at least three HSVd sequence variants in almond. One of the clones was found to be identical to the peach isolate AF (Sano et al., 1989), renamed as HSVd.p2 by Bussière et al. (1996). The other two sequence variants (HSVd.alm1 and HSVd.alm2) have not been described previously. The variants were named following the rules proposed by Bussière et al. (1996), with the three letter code introduced by Kofalvi et al. (1997) (i.e., HSVd.alm[number] for the almond variants). HSVd.alm1 and HSVd.alm2 are very closely related to HSVd.p2 (Figure 3). Both had a deletion of one 'A' in the stretch of seven 'A' between positions 39 and 45 of HSVd.p2. In addition, HSVd.alm2 had an 'A' to 'G' change at position 26. Keese and Symons (1985) described that the genome of typical viroids contains five structural domains, being sequence variation between strains mostly localized in the pathogenicity (P) and variable (V) domains. The changes of HSVd.alm1 and HSVd.alm2 are located at residues variable among HSVd sequences, in loops on the left part of the rod-like molecule that includes the P domain (Kofalvi et al., 1997), and within the positions at which HSVd.p2 differs from the HSVd reference sequence (HSVd.h1) (marked as arrowheads in Figure 3).

Multiple alignments with all the available HSVd sequences, including the two almond sequence variants characterized in this report, were obtained using the Clustal W program (Thompson et al., 1994) (not shown). Subsequent phylogenetic analyses were

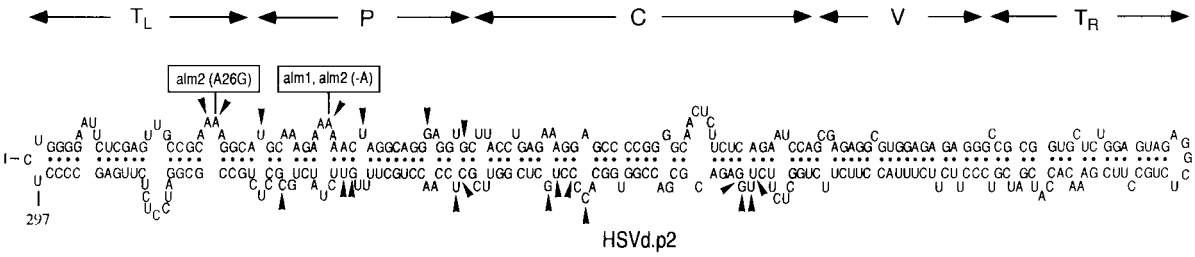

Figure 3. The nucleotide sequence and secondary structure of the reference variant HSVd.p2 and sequence variations observed in the two new almond variants, HSVd.alm1 and HSVd.alm2. The five domains proposed by Keese and Symons (1985) are indicated on top of the figure: C (central), P (pathogenic), V (variable), T_L (terminal left), and T_R (terminal right). Positions of the HSVd.p2 that differ from the HSVd reference sequence HSVd.h1 are shown by arrows.

carried out using the programs of the PHYLIP 3.5 c package (Felsenstein, 1993), as described previously (Kofalvi et al., 1997). The phylogenetic tree obtained (Figure 4), based on genetic distances, did not alter significantly the previously proposed clustering of HSVd sequences into five groups (Kofalvi et al., 1997). HSVd.alm1 and HSVd.alm2 were included within the group containing the reference sequence HSVd.p2, that together with the group containing citrus sequences have been postulated to cause the other phylogenetic groups to originate by genetic recombination (Kofalvi et al., 1997). Interestingly, the new variants cluster with HSVd.p2 albeit contain changes at the so called informative positions number 2 and 4 (A26→G and deletion of A44, respectively, as compared to HSVd.p2), not previously found in this phylogenetic group (see Figure 3 in Kofalvi et al., 1997). The first change (A26→G) had been observed only in some variants (c2, cit1 and cit2) belonging to the citrus group, while the second (deletion of A44, showing a six 'A' stretch in the P domain) opposes to the seven 'A' stretch that previously seemed characteristic of HSVd.g2-g3-g9, HSVd.apr2-apr5, and HSVd.p2 groups (Kofalvi et al., 1997). A summary of sequence characterizations of HSVd from *Prunus* spp. (Sano et al., 1989; Kofalvi et al., 1997, present study) reflects that 26 independent clones from plum, peach, apricot and almond have been sequenced so far; six of these clones (from plum, peach and almond) correspond to HSVd.p2 whereas seven (HSVd.p3, HSVd.apr3, HSVd.apr6, HSVd.apr7, HSVd.apr8, HSVd.alm1 and HSVd.alm2) contain no more than three changes as compared to HSVd.p2. All these sequences conform an independent cluster in the HSVd tree (Figure 4) and also indicate that variant sequences related to the HSVd.p2 are particularly well adapted to the infection of these plant species.

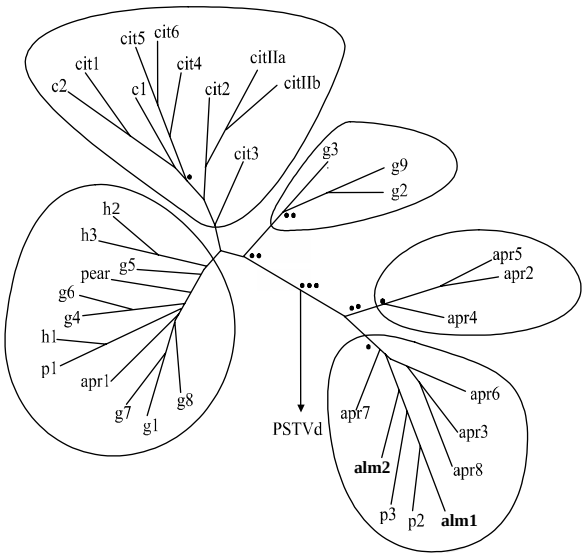

Figure 4. Phylogenetic tree of HSVd sequence variants. Phylogenetic analysis was based on genetic distances (PHYLIP 3.5 c package) calculated between all available HSVd sequence variants. The almond variants sequenced in this work are labelled alm1 and alm2. The position and branching of PSTVd, included in the analysis as an outgroup, is indicated by an arrow. The five phylogenetic groups identified are delineated. Dots near nodes indicate the statistical value of the nodes as determined by bootstrap analysis (100 replicates): ● ● ●, node detected in 100% of replicates; ●●, node detected in 80–100% of replicates; ●, node detected in >50% of replicates.

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References

Astruc N, Marcos JF, Macquaire G, Candresse T and Pallás V (1996) Studies on the diagnosis of hop stunt viroid in fruit trees: Identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents. Eur J Plant Pathol 102: 837–846

Bussière F, Lafontaine D and Perreault JP (1996) Compilation and analysis of viroid and viroid-like RNA sequences. Nucleic Acids Res 24: 1793–1798

Cañizares MC, Marcos JF and Pallás V (1998) Studies on the incidence of hop stunt viroid in apricot trees (*Prunus armeniaca*) by using an easy and short extraction method to analyze a large number of samples. Acta Horticult 472: 581–585

Diener TO (1987) The Viroids. New York: Plenum

Diener TO, Smith D, Hammond R, Albanese G, La Rosa R and Davino M (1988) Citrus B viroid identified as a strain of hop stunt viroid. Plant Disease 72: 691–693

Dinter-Gottlieb G (1986) Viroids and virusoids are related to group I introns. Proc Natl Acad Sci USA 83: 6520–6524

Di Terlizzi B, Digiaro M and Savino V (1994) Preliminary studies on virus like diseases of almond. Acta Horticult 373: 293–298

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle

Flores R, Di Serio F and Hernández C (1997) Viroids: the non-coding genomes. Sem Virol 8: 65–73

Flores R, Durán-Vila N, Pallás V and Semancik J (1985) Detection of viroid and viroid-like RNAs from grapevine. J Gen Virol 66: 2095–2102

Haas B, Klanner A, Ramm K and Sänger HL (1988) The 7S RNA from tomato leaf tissue resembles a signal recognition particle RNA and exhibits a remarkable sequence complementarity to viroids. EMBO J 7: 4063–4074

Jakab G, Kiss T and Solymosy F (1986) Viroid pathogenicity and pre-rRNA processing: a model amenable to experimental testing. Biochem Biophys Acta 868: 190–197

Keese P and Symons RH (1985) Domains in viroids: evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. Proc Nat Acad Sci USA 82: 4582–4586

Kofalvi SA, Marcos JF, Cañizares MC, Pallás V and Candresse T (1997) Hop stunt viroid (HSVd) sequence variants from *Prunus* species: evidence for recombination between HSVd isolates. J Gen Virol 78: 3177–3186

Martelli GP and Savino V (1997) Infectious diseases of almond with special reference to the Mediterranean area. Bull. OEPP/EPPO Bull. 27 (accepted for publication)

Meduski CJ and Velten J (1990) PSTV sequence similarity to large rRNA. Plant Mol Biol 14: 625–627

Pallás V, Más P and Sánchez-Navarro J (1998) Detection of plant RNA viruses by non-isotopic dot-blot hybridization. In: G. Foster and S. Taylor (eds) Plant virus protocols: from virus isolation to transgenic resistance. (pp 471–478) Humana Press, Totowa, New Jersey

Pallás V, Navarro A and Flores R (1987) Isolation of a viroid-like RNA from hop different from hop stunt viroid. J Gen Virol 68: 3201–3205

Podleckis EV, Hammond RW, Hurtt SS and Hadidi A (1993) Chemiluminescent detection of potato and pome fruit viroids by digoxigenin-labeled dot blot and tissue blot hybridization. J Virol Methods 43: 147–158

Polivka H, Staub U and Gross HJ (1996) Variation of viroid profiles in individual grapevine plants: novel grapevine yellow speckle viroid 1 mutants show alterations of hairpin I. J Gen Virol 77: 155–161

Sano T, Hataya T, Terai Y and Shikata E (1989) Hop stunt viroid strains from dapple fruit disease of plum and peach in Japan. J Gen Virol 70: 1311–1319

Semancik JS (1987) Viroids and viroid-like pathogens. CRC Press: Boca Raton

Semancik JS, Roistacher CN, Rivera-Bustamante R and Duran-Vila N (1988) Citrus cachexia viroid, a new viroid of citrus: relationship to viroids of the exocortis disease complex. J Gen Virol 69: 3059–3068

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

Shikata E (1990) New viroids from Japan. Sem Virol 1: 107–115

Solymosy F and Kiss T (1985) Viroids and snRNAs. In: Subviral Pathogens of Plants and Animals: Viroids and Prions. (pp 183–199) Academic Press, New York

Thompson JD, Higgins DG and Gibson TJ (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680