European Journal of Plant Pathology **102:** 837–846, 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

Nathalie Astruc[1], Jose F. Marcos[1], Guy Macquaire[2], Thierry Candresse[2] and Vicente Pallás [1,3]
[1]*Centro de Edafología y Biología Aplicada del Segura – CSIC, Murcia, Spain;* [2]*Station de Pathologie Végétale – INRA, Villenave d'Ornon, France;* [3]*Author for correspondence: Dr. Vicente Pallás, Dept. Mejora y Patología Vegetal, Centro de Edafología y Biología Aplicada del Segura – CSIC, Avda. Fama – 1, 30.003 – Murcia, Spain (Fax: 34-68-26 66 13)*

Accepted 13 June 1996

Key words: digoxigenin-labelled RNA probe, viroid diagnosis, viroid extraction methods, non-isotopic detection

Abstract

A non-radioactive digoxigenin-labelled RNA probe specific for hop stunt viroid (HSVd) diagnosis has been developed. The high sensitivity and specificity of this RNA probe in dot blot hybridizations to nucleic acids from field samples, allowed the confirmation of the presence of HSVd in apricot (*Prunus armeniaca* L.) and its detection in two fruit tree species not previously described as hosts of this pathogen, almond (*Prunus dulcis* Miller) and pomegranate (*Punica granatum* L.). This result supports and extends the notion of the world wide distribution of HSVd, infecting cultivated fruit trees. HSVd was also found to accumulate to much higher levels in mature apricot fruits than in leaves. Additionally, a sample processing procedure which does not involve the use of organic solvents was demonstrated to render faithful results when used for viroid detection. The combined reliability and facility of use of both this extraction procedure and the non-radioactive probe will benefit agronomic investigations addressing the detection and eradication of HSVd. Other applications of the work described here, as the study of possible causal relations between specific disorders and HSVd infection, are also discussed.

Introduction

Viroids are subviral plant pathogens whose genome consists of a small (240–370 nucleotide residues) circular RNA with a high degree of self complementarity in its sequence (Diener, 1991). In some well characterized examples it has been demonstrated that viroid infection causes plant diseases which affect agronomic quality. Hop stunt viroid (HSVd), as its name indicates, was first described as the causal agent of the stunt disease of hops in Japan, but since then it has been found in several plant species, most of them fruit trees like citrus, pear, peach or plum (Shikata, 1990). These plants either showed specific disorders or were latently infected. The diseases known as cachexia of citrus (Diener et al., 1988; Semancik et al., 1988; Levy and Hadidi, 1993) and dapple fruit of plums and peaches (Sano et al., 1989) have been associated with sequence variants of HSVd. Previous results indicated the presence of HSVd in an additional host, apricot, on the basis of detection of a viroid-like RNA of HSVd size and transmission to the experimental host cucumber (Flores et al., 1990; Di Serio et al., 1995). Additional studies are required to further evaluate the incidence of this viroid among fruit trees, as well as to investigate whether there is any correlation between other symptomatologies and HSVd presence in field samples. To reach this goal, easy and reliable diagnosis procedures will be undoubtedly welcome.

Since no viroid-encoded proteins have been identified, detection based on immunological techniques is not suitable for the diagnosis of viroids. Bioassays are not appropriate for screening large populations, mostly due to the requirements of greenhouse facilities and plant care. As an alternative, either nucleic acid hybridization (Owens and Diener, 1981), specific

double electrophoretic techniques (Schumacher et al., 1983; Flores et al., 1985), or approaches based on the amplification of sequences by the polymerase chain reaction (PCR) (Hadidi and Yang, 1990; Yang et al., 1992), have been developed for the detection of viroid RNAs. Double electrophoresis, although specific for small circular RNAs, presents the disadvantage of its relatively low sensitivity, while PCR, and to a lesser extent hybridization, requires costly equipment and reagents. Furthermore, hybridization initially relied on radioactively labelled probes which are non-desirable for routine use. A further improvement arose with the advent of non-radioactive methods for the detection of hybridized probes, allowing their progressive introduction as tools in the study of plant viruses (Habili et al., 1987; Eweida et al., 1990; Fouly et al., 1992; Gemmrich et al., 1993; Más et al., 1993; Dietzgen et al., 1994; Harper and Creamer, 1995; Sánchez-Navarro et al., 1996). Several reports have already described the use of these non-radioactive probes to detect viroid RNAs, albeit most of them correspond to potato spindle tuber viroid (PSTVd) (McInnes et al., 1989; Roy et al., 1989; Candresse et al., 1990; Hopp et al., 1991; Kanematsu et al., 1991; Welnicki and Hiruki, 1992; Podleckis et al., 1993; Singh et al., 1994). Recently, a non-radioactive tissue-print hybridization method was reported for viroid detection (Romero-Durbán et al., 1995), although the authors encountered problems in the detection of HSVd. On the other hand, most of the methods generally used for (viroid) RNA extraction require the use of phenol or other toxic organic solvents, making them undesirable for diagnosis laboratories that manage large numbers of samples.

In this work, we have developed a digoxigenin-labelled non-radioactive RNA HSVd probe that has allowed us to confirm and study the incidence of this viroid in apricot trees. Furthermore, making use of this sensitive probe, HSVd infection was found for the first time in two additional orchard crops: almond and pomegranate. Finally, we have adapted an extraction method that avoids the use of phenolics, previously described to prepare plant genomic DNA (Dellaporta et al., 1983), to obtain viroid-containing nucleic acids preparations suitable for diagnostic purposes.

Materials and methods

Plant material

Leaf samples from cultivars of apricot (*Prunus armeniaca* L.) and almond (*Prunus dulcis* Miller) were obtained from a collection kept at Centro de Edafología y Biología Aplicada del Segura (Murcia, Spain). Additionally, two apricot samples were collected from field trees at Caserta and Ugento (Italy). Material for experiments carried out to compare HSVd titer in leaves and fruits of apricot cv. 'Bulida' was kindly provided by Dr. G. Llácer (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain). Leaf samples from cultivars of pomegranate (*Punica granatum* L.) were obtained from Escuela Politécnica Superior de Orihuela (Alicante, Spain). A positive control of HSVd infection was provided by Dr. J. C. Desvignes (Centre Technique Interprofessionnel des Fruits et Légumes, Pringonrieux, France), and consisted of leaves from greenhouse-grown GF 305 peach (*Prunus persica* L.) inoculated under experimental conditions with the HSVd isolate causing the plum dapple fruit disease (Sano et al., 1989).

Nucleic acid extraction from tissue samples

Processing of frozen leaves was based on 'standard' methods for the extraction of viroid RNAs (Pallás et al., 1987). Briefly, 10 g leaf samples were homogenized in 40 ml of neutralized (pH 7, with NaOH) water-saturated phenol, 10 ml of 0.2 M Tris-HCl pH 8.9, 2.5 ml of 0.1 M (ethylenedinitrilo)tetraacetic acid (EDTA) pH 7.0, 2.5 ml of 5% (w/v) sodium dodecylsulfate (SDS) and 0.6 ml of mercaptoethanol (MCE). The homogenate was then centrifuged at 7700 $\times$ *g* for 15 min to separate phases. Phenolic phases were re-extracted with the non-phenolic components of the homogenization buffer, and the combined aqueous phases were finally re-extracted with neutralized water-saturated phenol. The final aqueous phases were enriched for small RNAs with a high content in secondary structure by chromatography through non-ionic cellulose CF-11 (Whatman, England) (Franklin, 1966) as previously described (Flores et al., 1985). The nucleic acids recovered in the final chromatographic elution volume were ethanol precipitated and resuspended in sterile water (1 ml/10 g tissue).

In addition to this 'standard' method, four other procedures for the extraction of nucleic acids were evaluated. (a) The first one is based on the quick action

of denaturants such as urea and Triton X-100 and is a slight modification of that previously used for the diagnosis of peach latent mosaic viroid (Ambrós et al., 1995). Two g of leaf tissue were homogenized in 10 ml 0.2 M Tris-HCl pH 8.9, 0.1 M NaCl, containing 100 μl 10% Triton X-100, 20 μl mercaptoethanol and 0.6 g urea. After centrifugation at 7700 × *g* for 20 min, the supernatant was saved and 1.25 ml 5% (w/v) SDS, 1.25 ml 0.1 M EDTA pH 7.0, and 20 ml of neutralized water-saturated phenol were added. The mixture was shaken for 5 min and then centrifuged at 7700 × *g* for 20 min. The nucleic acids in the aqueous phase were ethanol precipitated. (b) The second method is based on a previously described protocol for the extraction of plant genomic DNA (Dellaporta et al., 1983), which has been already used to enrich in viroid-like RNAs samples of partially purified nucleic acids (Pallás et al., 1987). Two g of leaf tissue were homogenized in 10 ml of 0.1 M Tris-HCl pH 8.0, 50 mM EDTA, 0.5 M NaCl and 10 mM MCE. After homogenization, 0.5 ml of 20% SDS were added and the mixture was incubated at 65 °C for 20 min. Subsequently, 2.5 ml of 5 M potassium acetate were added and incubation proceeded at 0 °C for another 20 min. Samples were centrifuged at 12000 × *g* for 15 min and the nucleic acids in the supernatant were ethanol precipitated. (c) The third method is based on direct extraction in a formaldehyde buffer to bypass the subsequent denaturation with this chemical prior to hybridization (Querci et al., 1995). Two g of leaf tissue were homogenized in a mixture of 2 ml of 37% formaldehyde, 2 ml 10X SCC (see below for 1X SSC composition) and 6 ml neutralized water-saturated phenol. Samples were centrifuged at 12000 × *g* for 15 min and the nucleic acids in the aqueous phase were ethanol precipitated. (d) Finally, a fourth procedure which previously was proved to be suitable for the extraction of PSTVd from tomato and subsequent analysis with non-radioactive probes (Roy et al., 1989) was used. Two g of leaf tissue were homogenized in 6 ml 0.1 M sodium acetate pH 9.0 plus 60 μl 10% Triton X-100. Following homogenization, 12 ml of neutralized water-saturated phenol were added, the mixture was shaken and finally centrifuged at 12000 × *g* for 15 min. The nucleic acids in the aqueous phase were ethanol precipitated.

Construction of a HSVd specific cDNA clone

A sample of low molecular weight RNAs obtained from a Spanish HSVd-infected peach cv. 'Jerónimo Copia' was provided by Dr. R. Flores (Instituto de Biología Molecular y Celular de Plantas, Valencia, Spain) and used as viroid source for cDNA synthesis. This sample was heated to 90 °C for 3 min in the presence of the HSVd-specific 26-mer oligonucleotide VP-19 (5′-dGCCCCGGGGCTCCTTTCTCAG-GTAAG-3′, complementary to HSVd residues 60–85, and encompassing the unique endonuclease restriction site Sma I, underlined, at residue 80 in the HSVd sequence), and slowly cooled to room temperature. The mixture was then subjected to reverse transcription (RT) with avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, USA) at 42 °C for 45 min, in a 20 μl volume. One-tenth of the RT product was directly subjected to PCR amplification, in a 50 μl volume reaction, in the presence of oligonucleotides VP-19 and the 27-mer VP-20 (5′-dCGCCCGGGGCAACTCTTCTCAGAATCC-3′, which contains HSVd residues 78–102, with the Sma I site underlined, and two additional unrelated residues at its 5′ end). Both primers lie in the strictly conserved central region of HSVd (Shikata, 1990). PCR cycling parameters were: denaturation at 94 °C for 1 min, followed by 30 cycles of 94 °C for 40 sec, 60 °C for 40 sec and 72 °C for 1 min, to finish with extension at 72 °C for 5 min. Electrophoretic analysis confirmed the presence of a monomeric PCR product of the expected size. This PCR product was phenol-extracted, ethanol-precipitated and digested with endonuclease Sma I following standard procedures (Sambrook et al., 1989). The resulting DNA fragment was cloned at the Sma I restriction site of the commercially available digested and dephosphorylated pUC18 plasmid (Pharmacia Biotech, Uppsala, Sweden). Several positive clones, some of them with head to tail dimeric insertions, were identified by restriction analysis. From one of these dimeric clones, an approximately 270 residues-long Eco RI-Bam HI restriction fragment was obtained and cloned into pBlueScript KS + (Stratagene, San Diego, USA), previously digested with the same two restriction enzymes, to yield pHSVd.EB (Figure 1a).

In vitro transcription of digoxigenin-labelled RNA probes

Approximately one microgram of pHSVd.EB was linearized with Eco RI (or, alternatively, with Bam HI when the plasmid was to be transcribed by the T3 polymerase), phenol-extracted and ethanol-precipitated. In order to obtain viroid complementary riboprobes, *in vitro* transcription was carried out with T7 RNA polymerase following the directions of the digoxigenin

RNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Transcript RNA was recovered by phenol extraction and ethanol precipitation, resuspended in freshly autoclaved water and used in the hybridization solution. We have stored digoxigenin RNA probes in ethanol at −20 °C for 4–5 months.

Dot-blot hybridization and detection of digoxigenin-labelled nucleic acids

Sample blotting onto membranes and hybridization were carried out essentially as described previously (Pallás et al., 1996). Briefly, samples were denatured with 7.4% formaldehyde in the presence of 6X SSC (White and Bancroft, 1982) and 4 μl spots and serial dilutions were applied to Hybond nylon membranes (Boehringer Mannheim, Mannheim, Germany). In some experiments, non-denatured controls were also applied. Membranes were air-dried and exposed to the UV light of a transilluminator for 3 min. Prehybridization was carried out at 68 °C for 2–3 h in 50% deionized formamide, 5X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 2% (w/v) blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.1% (w/v) N-laurylsarcosine and 0.01% (w/v) SDS. This solution was discarded and replaced by 100 μl/cm^2 of the same solution plus 0.1 μg/ml of the digoxigenin-labelled RNA probe and hybridization was allowed to proceed overnight at 68 °C. After hybridization, membranes were washed twice with 2X SSC, 0.1% SDS at room temperature for 5 min, and twice with 0.1X SSC, 0.1% SDS at 68 °C for 15 min. In order to detect the hybridized probe, binding to anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) and subsequent chemiluminiscent detection using CSPD (Boehringer Mannheim, Mannheim, Germany) as substrate were carried out as described (Más et al., 1993; Pallás et al., 1996). Films were exposed to the membranes for 10–60 min, except were indicated. Tissue-print experiments were carried out as described previously (Más and Pallás, 1995; Más and Pallás, 1996).

Results

Development of a non-radioactive RNA probe to diagnose HSVd

Initial electrophoretic analyses indicated the presence of HSVd among apricot and peach orchard trees from the regions of Valencia and Murcia (Flores et al., 1990; Flores et al., 1992; Marcos and Pallás, unpublished). As a first step to demonstrate and further evaluate the occurrence of HSVd, we decided to set up a non-radioactive hybridization procedure for the sensitive detection of this viroid. A nearly full-length cDNA corresponding to a Spanish peach HSVd isolate was cloned, as described above, under the control of two different phage transcriptional promoters to yield pHSVd.EB (Figure 1a). Subsequent sequencing confirmed the HSVd identity of the insert, revealing that the 278 residue-long fragment showed no sequence differences with the previously sequenced peach AF HSVd isolate from Japan (Sano et al., 1989). It is remarkable that the two isolates infecting the same plant species show such sequence conservation despite their distinct geographical origin.

Control experiments were conducted to evaluate the sensitivity and specificity as HSVd probe of the digoxigenin-labelled T7 pHSVd.EB transcript (Figure 1b). Serial dilutions of either T3 or T7 polymerase non-labelled pHSVd.EB transcripts, as well as of nucleic acids prepared following the standard extraction method from either uninfected or HSVd-infected GF305 peach controls, were dot-blotted onto nylon membranes and hybridized with the HSVd probe. As expected, the extract from infected plants showed a strong hybridization signal, even at 1/1000 dilution, while no signal could be detected in the non-infected control (Figure 1b, rows 4 and 3, respectively). The highest dilution at which infection was still detected corresponded to 12.5 ng of fresh weight tissue. Accurate measurements of the concentration of the non-labelled transcripts applied to the membrane allowed us to determine that the probe is able to detect subpicogram amounts of viroid RNAs, up to 10–100 fg in 2 hour-long exposures of the film (Figure 1b, row 1). As an additional demonstration of the high stringency of the hybridization conditions (68 °C and 50% formamide) it should be noted that the minus sense RNA probe hybridized only to a very minor extent with the transcript of the same polarity (Figure 1b, row 2); viroid RNAs, as corresponds to their typical self-complementary structure, are capable of partial

self-annealing. Furthermore, no signal was observed with extracts from plants infected by two other unrelated viroids, citrus exocortis (CEVd) (Figure 1b, row 5) and avocado sunblotch (ASBVd) (not shown) viroids. Finally, a comparison experiment was conducted in which a duplicate of the membrane shown in Figure 1b was hybridized to a ^{32}P-labelled RNA probe specific for HSVd (not shown). No significant differences regarding sensitivity and specificity were found between both labelling procedures, thus further stressing the suitability of digoxigenin probes for diagnostic purposes.

HSVd detection in apricot samples from different sources

The non-radioactive hybridization procedure described above was used to conduct an initial survey of viroid incidence in apricot samples from Spain and Italy. A number of clear positives corresponding to HSVd infection were found among samples from Murcia (Spain) and Caserta (Italy) (Figure 2a). The intensity of the signal was variable, always below that from the positive control of infected GF305 peach (compare with Figures 1b and 3a) and sometimes just above the detection limit of the analysis. The presence of negative samples (Figure 2a, cv. 'Baracca') served as an internal control on the reliability of our diagnosis. We must stress that most of the positive samples, including the ones with weak signals (i. e. low titer of HSVd), scored as negative when analysed by a double electrophoresis approach (not shown). It is generally recognized that HSVd reaches low concentrations in its host trees (Shikata, 1990), emphasizing the need for highly sensitive and specific detection procedures as the one described here. Most of the apricot trees infected with HSVd belong to cultivars of agronomic importance in the Murcia and Valencia areas, as is the case of 'Bulida d'Arques', 'Rojo de Carlet', 'Colorado' or 'Pepito del Rubio' (Figure 2a), 'Bulida' (Figure 2b), and also 'Real Fino' (not shown). In summary, six out of seven cultivars from Murcia (Spain) and one out of two from Italy, were found infected. Although some of these trees exhibited specific disorders, no clear correlation between symptomatology and viroid infection can be drawn at this point.

Additional experiments were conducted in which fruits from apricot cv. 'Bulida' were analysed together with their corresponding leaves (Figure 2b). It is important to stress that both types of samples were harvested at the same time. The result clearly showed that HSVd

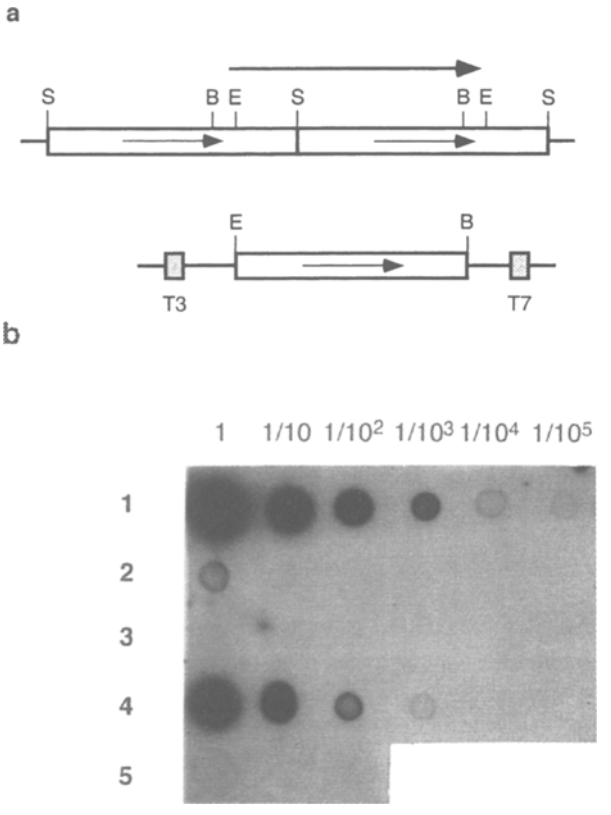

Figure 1. A digoxigenin-labelled RNA probe for HSVd detection. (a) Schematic diagram showing plasmid pHSVd.EB and its construction procedure. From a HSVd dimeric clone (top) an EcoR I – BamH I restriction fragment was obtained and directionally inserted into pBS/KS + to yield pHSVd.EB (bottom). Open boxes represent viroid sequences and the thin arrows inside reflect their orientation. Lines represent plasmid sequences. The thick arrow at the top spans the monomeric HSVd sequence from nucleotide 1 to 297 , while restriction sites and their position are indicated as follows: B (BamH I at position 272), E (EcoR I at position 6), and S (Sma I at position 80). Shaded boxes represent the T3 and T7 phage transcriptional promoters from which *in vitro* transcription is initiated. (b) Different nucleic acid preparations were analysed by dot-blot hybridization to the digoxigenin-labelled T7 transcript of pHSVd.EB. Samples analysed were: non-labelled T3 (1) or T7 (2) transcripts of pHSVd.EB, CF-11 cellulose-purified nucleic acids from either non-infected (3) or HSVd-infected (4) GF305 peach, and an equivalent nucleic acids extract from the experimental host *Gynura aurantiaca* infected with CEVd (5). Undiluted samples and five serial ten-fold dilutions were dotted onto the membranes. In the case of the non-labelled transcripts (rows 1 and 2), the first undiluted dot contains 1 ng of RNA, while in the peach extracts (rows 3 and 4) the first dot corresponds to 12.5 mg of tissue.

reaches much higher concentrations in mature fruits than in leaves (Figure 2b). The obvious implication is that, whenever possible, fruit samples should be analysed to guarantee the highest sensitivity in the detection of the viroid. To further investigate this

HSVd location, the tissue printing technique (Más and Pallás, 1995) was used to examine the spatial distribution of the viroid in fruits. One representative sample is shown (Figure 2c) to illustrate that, surprisingly, HSVd was not evenly distributed in fruits. However, this varying viroid concentration could not be correlated neither with patches of physiologically altered tissue nor with maturation areas.

HSVd presence in other orchard species

Our survey to detect HSVd infection was extended to other economically important crops in our growing areas (Figure 3). In an extensive series of experiments we found two additional tree species infected with HSVd which had not previously been reported as hosts of this viroid: pomegranate (*Punica granatum* L.) and almond (*Prunus dulcis* Miller). The four cultivars of pomegranate analysed showed clear positive signals (Figure 3b), revealing the presence of the viroid. We could not identify a single non-infected pomegranate which could be taken as internal negative control in this study. However, the high intensity of the hybridization signals, several-fold times above that of the negative samples found in other species (compare with Figures 1b, 2, 3a and 3c), strongly indicates HSVd infection. Moreover, subsequent electrophoretic analysis, as an additional control, showed the presence of a circular RNA with electrophoretic mobility close to that of HSVd in one of these pomegranate samples (not shown).

Two cultivars of almond, out of four analysed, were found infected with HSVd (Figure 3c). The weak signal in one of the samples indicated a low viroid titer, notorious when compared with peaches, apricots and pomegranates. Finally, it must be stressed that in our analysis HSVd infection was not found in several other cultivated trees, including hazels (*Corylus avellana*) (Figure 3a) and olives (*Olea europaea*) (not shown), which were grown in close proximity to infected almonds.

A procedure for viroid RNA extraction without the use of organic solvents

Considering the wide distribution of HSVd among orchard trees, a short, easy and reliable procedure to process the samples for routine diagnosis would be highly desirable, specially for those agronomic research stations working on the detection and eradication of this plant pathogen. In these laboratories, avoidance of the use of toxic organic solvents such as phenol would be beneficial. Up to this point, and following directions to enrich the nucleic acid samples given the low concentration that HSVd reaches in infected tissues (Shikata, 1990), we have used a 'standard' time-

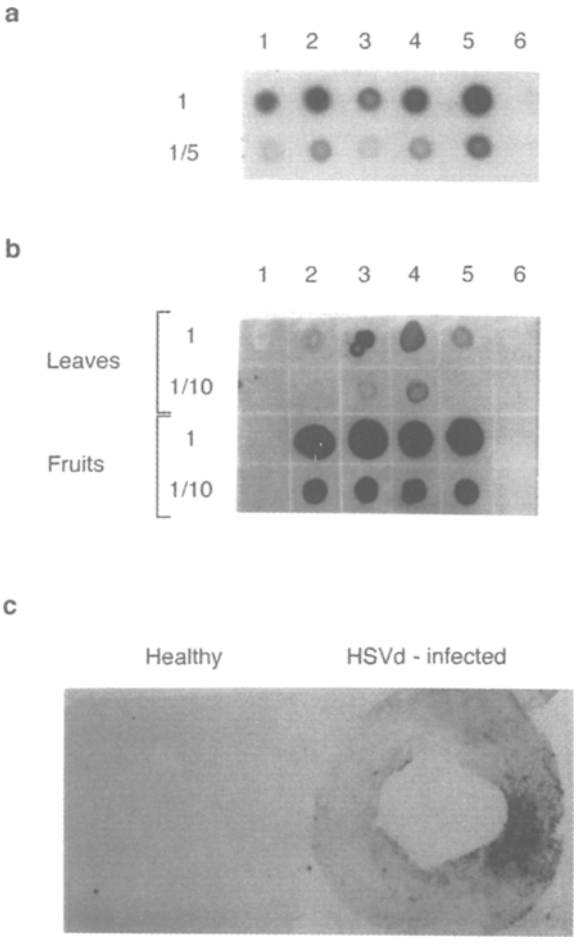

Figure 2. HSVd detection in apricot by hybridization to the digoxigenin-labelled HSVd-specific RNA probe. **(a)** Nucleic acid preparations from one leaf sample harvested in the field at Caserta (Italy) (1), as well as from leaf samples from the commercial cultivars grown in Murcia (Spain) 'Bulida d'Arques' (2), 'Rojo de Carlet' (3), 'Colorado' (4), 'Pepito del Rubio' (5) and 'Baracca' (6) were analysed by dot-blot hybridization. Undiluted samples and five-fold dilutions were dotted onto the membranes. **(b)** Nucleic acid preparations from leaf (top) and fruit (bottom) samples from six different trees (1 to 6) of the apricot commercial cultivar 'Bulida' were analysed; only four of them were found to be infected (2 to 5). Undiluted samples and ten-fold dilutions were dotted onto the membranes. Note that samples in the same column correspond to the same tree and were harvested at the same time. **(c)** Half fruits from uninfected (left) and HSVd-infected (right) trees of the apricot cultivar 'Bulida', were tissue-printed onto membranes and analysed by hybridization. Visual inspection of the fruit pigments transferred to the membrane confirmed that 'printing' was carried out evenly.

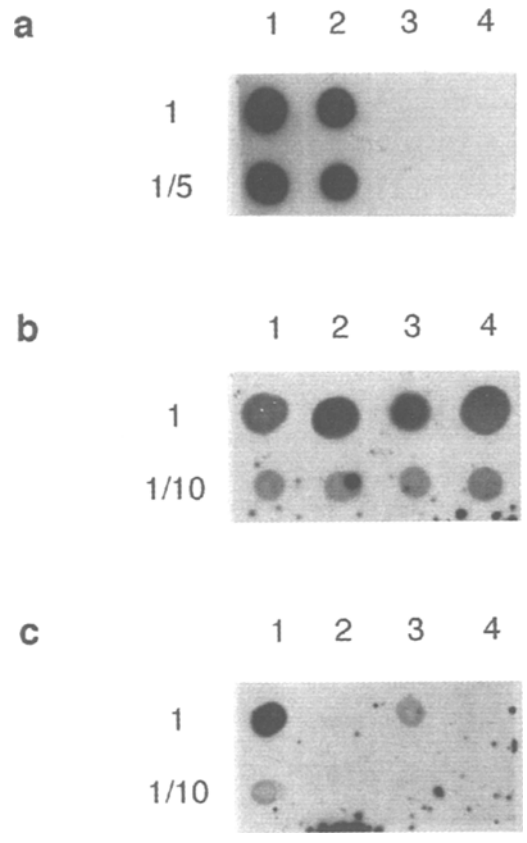

Figure 3. HSVd detection in different fruit trees by hybridization to the digoxigenin-labelled HSVd-specific RNA probe. **(a)** Nucleic acid preparations from leaf samples of the positive control of HSVd-infected GF305 peach (1 and 2, in duplicate), and two different field samples of hazels cv. 'Negret' (3 and 4) were analysed. Undiluted samples and five-fold dilutions were dotted onto the membranes. **(b)** Nucleic acid preparations from leaf samples of pomegranate experimental cultivars 'PTO 4' (1), 'ME 15' (2), 'PTO 1' (3), and 'BA 1' (4) were analysed. **(c)** Nucleic acid preparations from leaf samples of almond cultivars 'Non pareil' (1), 'Ferraduel' (2), 'Tuono' (3), and 'Ardéchoise' (4) were analysed. In (b) and (c) undiluted samples and ten-fold dilutions were dotted onto the membranes. In all the cases, samples were analysed by dot-blot hybridization.

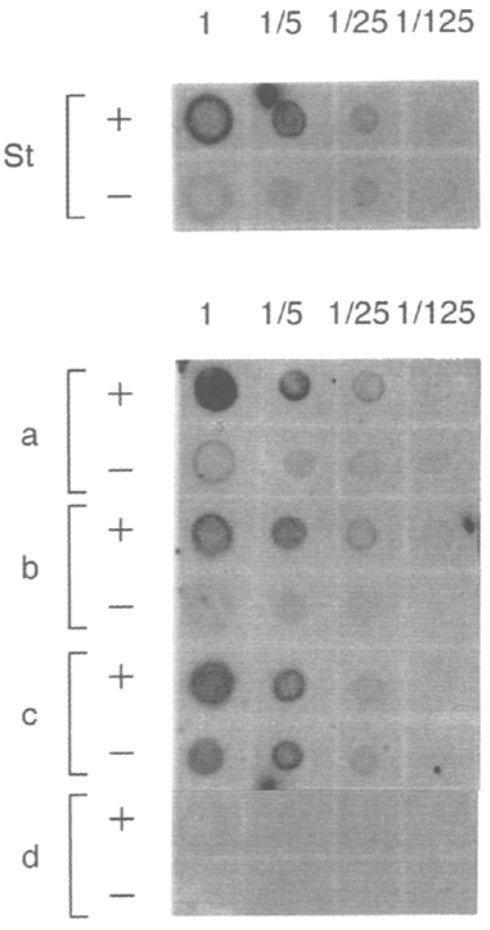

Figure 4. Evaluation of different procedures for nucleic acid extraction and HSVd detection. Leaf samples from HSVd-infected peach cv. 'Jerónimo' were extracted following the five procedures described in the Materials and Methods section as 'standard' (**St**) and 'a' to 'd' (**a–d**). All the nucleic acid extracts were ethanol precipitated, resuspended in water and directly analysed by dot-blot hybridization (note that for the standard procedure no further purification through CF-11 cellulose was done, unlike the previous experiments). Undiluted samples and five serial five-fold dilutions were dotted onto the membranes. (+) and (−) indicate samples that were either formaldehyde denatured or non-denatured, respectively, prior to dotting onto the membranes.

consuming extraction protocol that includes phenolics and non-ionic cellulose chromatography. We hypothesized that we could simplify the extraction procedure and still, given the high sensitivity and specificity of the probe, obtain faithful hybridization results. Four sample processing methods were evaluated for viroid RNA extraction (see materials and methods section). A single experiment is shown (Figure 4) which represents the results of four repetitions, carried out on apricot and peach tissues. Three procedures were suitable for viroid detection, giving similar results in terms of level of the signal (Figure 4, standard and a-c). Interestingly, one protocol is based upon a procedure used for the extraction of plant genomic DNA which does not make use of toxic organic solvents (Figure 4b). Also noteworthy is the fact that formaldehyde denaturation, either in the homogenization step or as denaturant previous to dotting of samples on the membranes, is necessary to obtain intense signals. Surprisingly, a slight variation of a procedure previously reported to yield good results in the detection of PSTVd in

tomato (Roy et al., 1989) did not work with the non-herbaceous species analysed here (Figure 4d).

Discussion

The use of non-radioactive, versus radioactively-labelled, nucleic acid probes for hybridization studies is becoming a suitable alternative to radioactivity. This is specially true not only for non-specialized laboratories but also for those dealing with continuous analyses of large numbers of samples. A typical application of this non-isotopic approach is the detection of pathogens. In this emerging scenario, we report here the development of a non-radioactive digoxigenin-labelled RNA probe for the diagnosis of the widely distributed viroid HSVd.

The high specificity of the HSVd probe, together with the hybridization conditions reported, is illustrated by the absence of signal in control samples non-infected or infected by unrelated viroids (Figure 1b). Regarding sensitivity, the nearly full-length probe described here performs as well as previous radioactive or multimeric probes described for other viroids (Roy et al., 1989; Candresse et al., 1990; Kanematsu et al., 1991; Singh et al., 1994), clearly detecting subpicogram amounts of HSVd RNAs. HSVd was also detected in dilutions of infected plant extracts corresponding to 12.5 ng of analysed tissue (Figure 1b). Experiments conducted in parallel showed that with the use of a ^{32}P-labelled RNA probe no additional signals were detected in higher dilutions of samples (i.e., there was no improvement in the detection limit). Our experience showed that the non-radioactive hybridization procedure resulted in the identification of HSVd infection in samples that appeared viroid-free when analysed by electrophoresis. Moreover, experiments with an RT-PCR approach showed that no PCR product appeared in any of the samples scored as free of viroid by hybridization to the digoxigenin probe (not shown).

In order to reduce and optimize handling of samples, we also evaluated several protocols for nucleic acid extraction from fruit tree leaves (Figure 4). Our results showed that, given the sensitivity and specificity of the probe, no viroid enrichment with CF-11 cellulose was necessary to obtain a reliable result. We also demonstrated that, as described for other viroids (Flores, 1986), formaldehyde denaturation is necessary in order to obtain intense signals. Several different protocols gave positive signals of aproximately the same intensity (Figure 4 a-c). Among them, a short procedure based on genomic DNA extractions (Dellaporta et al., 1983) which was previously used in the purification of double stranded viral RNAs (De Paulo and Powell, 1995) and also to enrich partially purified extracts in viroid-like RNAs (Pallás et al., 1987). This procedure, which does not use organic solvents, gave a response as good as the classical viroid extraction protocol (compare rows 'b' and 'St' in Figure 4). We propose, therefore, the use of this easy and short extraction procedure coupled to the digoxigenin RNA probe for routine diagnosis of HSVd.

Going beyond the description of a HSVd probe, we have made use of this tool to detect HSVd by molecular hybridization in apricots and to demonstrate HSVd infection in two additional crops, almond and pomegranate, not previously shown as hosts of this pathogen (Figures 2 and 3). In doing so, we also demonstrated the reliability of this procedure to detect viroid infection in field samples. Several commercially important apricot cultivars were found to be infected with HSVd. Although in the initial description of HSVd infection in apricots a possible correlation with the disease known as 'viruela' was considered (Flores et al., 1990), this hypothesis seems unlikely today since the viroid has been also found in asymptomatic fruits (R. Flores and G. Llácer, pers. comm.). Some of the apricot samples in our study exhibited alterations in leaves (vein clearings and patches of yellow tissue) and/or fruits (etchings and 'viruela' spots) albeit it is not yet possible to draw any conclusion on a possible viroid etiology. Future studies will be necessary in this direction, including more extensive surveys to establish correlation between disease and viroid presence and, ultimately, controlled inoculation experiments onto healthy plants. The same conclusion holds for the other two newly described HSVd hosts: almond and pomegranate. Even if HSVd turns out to be latent in these species, efforts should be addressed to the eradication of this pathogen, thus avoiding the establishment of field reservoirs that, potentially, could be transmitted to susceptible crops. It must be remembered that sequence variants of HSVd have been associated with the cachexia disease in citrus (Diener et al., 1988; Semancik et al., 1988; Levy and Hadidi, 1993) and the dapple fruit in peach and plum (Sano et al., 1989), and that these species are cultivated close to apricots in several areas around the world. We hope that the diagnosis procedures described here will substantially help future detection, diagnosis and eradication programs.

Finally, future lines of research are evident from this work. The ongoing sequencing of all the HSVd new isolates will expand the phylogenetic tree of HSVd variants, encompassing sequences from up to ten plant species and locations from all around the world. Thus, the HSVd natural system offers a unique opportunity for the study in viroids of the genetic determinants of host identity as well as of the divergence due to evolutionary history. In this regard, several so-called informative sequence changes have already been mentioned for HSVd citrus variants (Hsu et al., 1994), and is therefore likely the identification of sequences specific for other genera able to host HSVd infection.

Acknowledgements

We thank Dr R. Flores for critical reading of the manuscript and Dr. N. Durán-Vila for helpful comments. We also thank J. Martinez-Fresneda for her technical assistance. Finally, we thank Drs. E. García and P. Melgarejo for providing the almond and pomegranate samples and Dr. J. Aramburu for the hazel samples. This research was supported by grant AIR3-CT93-1567 from the European Commission.

References

Ambrós S, Desvignes JC, Llácer G and Flores R (1995) Peach latent mosaic and pear blister canker viroids: Detection by molecular hybridization and relationships with specific diseases affecting peach and pear trees. Acta Horticulturae 386: 515–521

Candresse T, Macquaire G, Brault V, Monsion M and Dunez J (1990) ^{32}P- and biotin-labelled *in vitro* transcribed cRNA probes for the detection of potato spindle tuber viroid and chrysanthemum stunt viroid. Res Virol 141: 97–107

De Paulo J and Powell C (1995) Extraction of double stranded RNA from plant tissues without the use of organic solvents. Plant Dis 79: 246–248

Dellaporta S, Wood J and Hicks J (1983) A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1: 19–21

Di Serio F, Ragozzino A and Alioto D (1995) Viroid-like RNAs associated with some apricot fruit disorders. Acta Horticulturae 386: 551–553

Diener T (1991) Subviral pathogens of plants: Viroids and viroidlike satellite RNAs. FASEB J 5: 2808–2813

Diener T, 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 Dis 72: 691–693

Dietzgen R, Xu Z and Teycheney P (1994) Digoxigenin-labelled cDNA probes for the detection of two potyviruses infecting peanut (*Arachis hypogaea*). Plant Dis 78: 708–711

Eweida M, Singh R and Abounaidar M (1990) Comparison between ELISA and biotin-labelled probes from cloned cDNA of potato virus X for the detection of virus in crude tuber extracts. Plant Pathol 30: 623–628

Flores R (1986) Detection of citrus exocortis viroid in crude extracts by dot-blot hybridization: Conditions for reducing spurious hybridization results and for enhancing the sensitivity of the technique. J Virol Meth 13: 161–169

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

Flores R, Hernández C, Avinent L, Hermoso A, Llácer G, Juarez J, Arregui JM, Navarro L and Desvignes JC (1992) Studies on the detection, transmission and distribution of peach latent mosaic viroid in peach trees. Acta Horticulturae 309: 325–330

Flores R, Hernández C, García S and Llácer G (1990) Is apricot "viruela" (pseudopox) induced by a viroid? In: Proceedings of the 8th Congress of the Mediterranean Phytopathological Union, Agadir

Fouly H, Domier L and D'Arcy C (1992) A rapid chemiluminiscent detection of barley yellow dwarf virus. J Virol Meth 39: 291–298

Franklin R (1966) Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc Natl Acad Sci USA 55: 1504–1511

Gemmrich A, Link G and Seidel M (1993) Detection of grapevine fanleaf virus (GFLV) in infected grapevines by non-radioactive nucleic acid hybridization. Vitis 32: 237–242

Habili N, McInnes J and Symons R (1987) Non-radioactive, photobiotin-labelled DNA probes for the routine diagnosis of barley yellow dwarf virus. J Virol Meth 16: 225–237

Hadidi A and Yang X (1990) Detection of pome fruit viroids by enzymatic cDNA amplification. J Virol Meth 30: 261–270

Harper K and Creamer R (1995) Hybridization detection of insect-transmitted plant viruses with digoxigenin-labelled probes. Plant Dis 79: 563–567

Hopp HE, Hain L, Almonacid FB, Tozzini AC, Orman B, Arese AI, Ceriani MF, Saladrigas MV, Vas MD and Mentaberry AN (1991) Development and application of a nonradioactive nucleic acid hybridization system for simultaneous detection of four potato pathogens. J Virol Meth 31: 11–30

Hsu Y, Chen W and Owens RA (1994) Nucleotide sequence of a hop stunt viroid variant isolated from citrus growing in Taiwan. Virus Genes 9: 193–195

Kanematsu S, Hibi T, Hashimoto J and Tsuchizaki T (1991) Comparison of nonradioactive cDNA probes for detection of potato spindle tuber viroid by dot-blot hybridization assay. J Virol Meth 26: 189–197

Levy L and Hadidi A (1993) Direct nucleotide sequencing of PCR-amplified DNAs of the closely related citrus viroids IIa and IIb (Cachexia). In: Proceedings of the 12th Conference of the International Organization of Citrus Virologists, Riverside, pp. 180–186

Más P and Pallás V (1995) Non-isotopic tissue-printing hybridization: a new technique to study long-distance plant virus movement. J Virol Meth 52: 317–326

Más P and Pallás V (1996) Long-distance movement of cherry leaf roll virus in infected tobacco plants. J Gen Virol 77: 531–540

Más P, Sánchez-Navarro J, Sánchez-Pina M and Pallás V (1993) Chemiluminescent and colorigenic detection of cherry leaf roll virus with digoxigenin-labelled RNA probes. J Virol Meth 45: 93–102

McInnes J, Habili N and Symons R (1989) Non-radioactive, photobiotin-labelled DNA probes for routine diagnosis of viroids in plant extracts. J Virol Meth 23: 299–312

Owens R and Diener T (1981) Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid spot hybridization. Science 213: 670–672

Pallás V, Más P and Sánchez-Navarro J (1996) Detection of plant RNA viruses by non-isotopic dot-blot hybridization. In: Foster G and Taylor S (eds) Plant Virus Protocols: From Virus Isolation to Transgenic Resistance. Humana Press, Totowa (in press)

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 E, Hammond R, Hurtt S and Hadidi A (1993) Chemiluminiscent detection of potato pome fruit viroids by digoxigenin-labelled dot blot and tissue blot hybridization. J Virol Meth 43: 147–158

Querci M, Owens RA, Vargas C and Salazar LF (1995) Detection of potato spindle tuber viroid in avocado growing in Peru. Plant Dis 79: 196–202

Romero-Durbán J, Cambra M and Durán-Vila N (1995) A simple imprint-hybridization method for detection of viroids. J Virol Meth 55: 37–47

Roy B, AbouHaidar M and Alexander A (1989) Biotinylated RNA probes for the detection of potato spindle tuber viroid (PSTV) in plants. J Virol Meth 23: 149–156

Sambrook J, Fritsch E and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sánchez-Navarro J, Cano E and Pallás V (1996) Non-radioactive molecular hybridization detection of carnation mottle virus in infected carnations and its comparison to serological and biological techniques. Plant Pathol 45: 375–382

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

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

Semancik J, Roistacher C, Rivera-Bustamante R and Durán-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

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

Singh R, Boucher A, Lakshman D and Tavantzis S (1994) Multimeric non-radioactive cRNA probes improve detection of potato spindle tuber viroid (PSTVd). J Virol Meth 49: 221–234

Welnicki M and Hiruki C (1992) Highly sensitive digoxigenin-labelled DNA probe for the detection of potato spindle tuber viroid. J Virol Meth 39: 91–99

White B and Bancroft F (1982) Cytoplasmic dot hybridization. J Biol Chem 257: 8569–8572

Yang X, Hadidi A and Garnsey S (1992) Enzymatic cDNA amplification of citrus exocortis and cachexia viroids from infected citrus hosts. Phytopathology 82: 279–285