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Indexing of citrus viroids by imprint hybridisation

A. Palacio-Bielsa, X. Foissac and N. Duran-Vila*
*Departamento de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias, Apartado Oficial, 46113 Moncada (Valencia) Spain; *Author for correspondence (Fax: +34-96-139-0240; E-mail: nduran@ivia.es)*

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Abstract

A method based on the hybridisation of tissue imprints was developed for routine indexing of citrus viroids. For maximum sensitivity and reliability, the inoculation of *Citrus medica* (Etrog citron) as a viroid amplification host is required. Hybridisation against Digoxigenin-labelled RNA- or DNA-probes followed by detection of viroid-probe hybrids using anti-DIG-alkaline phosphate conjugate and the chemiluminescence substrate CSPD was suitable for the detection of all citrus viroids with the same sensitivity as other available methods. The overall process is extremely simple and allows quick analysis of large numbers of samples by easily trained personnel and minimum equipment.

Viroids are infectious single-stranded circular RNA molecules which lack a capsid protein and mRNA activity. Viroids are the smallest plant pathogens and cause diseases in economically important crops. Commercial citrus harbour several viroids (Duran-Vila et al., 1988), which have been characterised as variants of five different viroids species (Flores et al., 1998). Citrus exocortis viroid (CEVd) with numerous variants reported (Gross et al., 1982; Visvader and Symons, 1985) and specific citrus variants (referred as CVd-IIb) of hop stunt viroid (HSVd) are causal agents of the exocortis (Semancik and Weathers, 1972) and cachexia (Semancik et al., 1988) diseases, respectively. Three additional viroids (CVd-I, CVd-III and CVd-IV) and another HSVd variant (referred to as CVd-IIa) are known to induce specific symptoms on Etrog citron (*Citrus medica* L.) (Duran-Vila et al., 1988) and CVd-I and CVd-III affect trees grown on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock (Roistacher et al., 1993; Semancik et al., 1997). Old citrus cultivars from all over the world, are usually infected by several citrus viroids.

Viroid control is critical for the commercial propagation of budwood released from quarantine, sanitation and certification programmes. These programmes require the performance of large numbers of indexing tests which must be sensitive and economic.

Biological indexing of exocortis and cachexia is based on the observation of symptoms induced on two indicator plants, Etrog citron 861-S1 (Roistacher et al., 1977) and Parson's Special mandarin (Roistacher et al., 1973). For sensitive detection, the inoculation of 4–6 plants of Etrog citron and 4–8 plants of Parson's Special mandarins graft-propagated onto vigorous rootstocks has been recommended. Inoculated indicators must be incubated at 27–32 °C for 3–6 months and 9–18 months, respectively, before symptom observation. This procedure is very time consuming and expensive due to the additional costs of maintaining greenhouses at elevated temperatures.

Nucleic acid analysis of viroid-infected plants by electrophoresis, molecular hybridisation and RT-PCR have been proposed as alternatives to biological indexing (Owens and Diener, 1981; Flores, 1988; La Rosa et al., 1988; Sano et al., 1988; Yang et al., 1992). The sensitivity and reliability of these methods for routine indexing of citrus viroids depends on the concentration and distribution of the viroids in the host

plants. Citrus viroids are not evenly distributed in species other than citron (Hadas et al., 1989; Duran-Vila et al., 1991) and their concentrations vary considerably among species and environmental conditions (La Rosa et al., 1988; Davino et al., 1991; Palacio, unpublished results). Sequential polyacrylamide gel electrophoresis (sPAGE) analysis of inoculated citrons was proposed as the most sensitive alternative to the conventional methods based solely on biological indexing (Duran-Vila et al., 1993). In spite of the improvement in terms of sensitivity and cost, the manipulations associated with nucleic acid extraction have been the limiting factor in terms of the number of indexing tests to be performed at a given time.

Recent reports indicate that viroids can be detected by molecular hybridisation of imprinted membranes (Podleckis et al., 1993; Romero-Durbán et al., 1995). The technique has been successfully applied for the detection of CSVd (Duran-Vila et al., 1996). Here, modifications of this imprint hybridisation technique are described for the successful detection of all citrus viroids and a protocol that can be used for routine indexing is outlined.

The selection 861-S1 of Etrog citron budded onto rough lemon (*Citrus jambhiri* Lusk.) rootstock was used as a source of tissue. The citron plants were graft-inoculated from several viroid-infected sources maintained in the viroid collection of the Instituto Valenciano de Investigaciones Agrarias (IVIA). Inoculated plants and uninoculated controls were kept in a greenhouse at 28–32 °C and the tissue was analysed by nucleic acid extraction and 5% sPAGE (Duran-Vila et al., 1993) to verify infection. In all the preliminary assays, the viroid sources were: CEVd (variant CEVd-117), CVd-I (variant CVd-Ia), HSVd (variant CVd-IIa), CVd-III (variant CVd-IIId) and CVd-IV (Duran-Vila et al., 1988). Additional sources tested included other variants of these viroids as well as naturally-occurring field isolates containing several viroids.

Stems were freshly cut transversely or longitudinally into approximately 1-cm-long pieces and firmly pressed onto the surface of polyvinylidene difluoride (PVDF) (Immobilon^{N}-N, Millipore) or positively charged Nylon (Boehringer Mannheim) membranes. PVDF and Nylon membranes were used for chromogenic and chemiluminescence detection respectively. The imprinted samples were immobilised by UV cross-linking and were stored in the dark until processing.

Digoxigenin (DIG)-labelled probes were obtained using a cloned plasmid containing full-length viroid monomeric DNA ligated to *Eco* R-V restricted pT7-Blue vector (Novagen). Cloned viroid DNA sequences had been previously obtained by RT-PCR using five pairs of synthetic oligonucleotides complementary and homologous to the sequence of the upper strand of the C region of each viroid (Table 1), and were confirmed by sequence analysis. Full-length monomeric RNA probes complementary to the viroid sequence were

Table 1. Complementary and homologous oligodeoxyribonucleotide primers

Viroid	Primers[1]	Sequence
CEVd	Complementary (CEVd-c)	5′-CCGGGGATCCCTGAAGGA-3′
	Homologous (CEVd-h)	5′-GGAAACCTGGAGGAAGTCG-3′
CVd-I	Complementary (CVd-I-c)	5′-TTCGTCGACGACGACCAGTC-3′
	Homologous (CVd-I-h)	5′-GGCTCGTCAGCTGCGGAGGT-3′
CVd-II	Complementary (CVd-II-c)	5′-GCCCCGGGGCTCCTTTCTCAGGTAAG-3′
	Homologous (CVd-II-h)	5′-CGCCCGGGGCAACTCTTCTCAGAATCC-3′
CVd-III	Complementary (CVd-III-c)	5′-TTCGTCGACGACGACAGGTA-3′
	Homologous (CVd-III-h)	5′-GGCAGCTAAGTTGGTGACGC-3′
CVd-IV	Complementary (CVd-IV-c)	5′-GGGTAGTTTCTATCTCAG-3′
	Homologous (CVd-IV-h)	5′-GGTGGATACAACTCTTGGG-3′

[1]CEVd primers are complementary and homologous to nucleotides 81–98 and 99–117 of CEVd-C (Gross et al., 1982). CVd-I primers are complementary and homologous to nucleotides 84–103 and 104–123 of CVd-Ia (Semancik et al., 1997). CVd-II primers are complementary and homologous to nucleotides 60–85 and 78–102 of HSVd (Ohno et al., 1983) with the modification described by Astruc et al., (1996). CVd-III primers are complementary and homologous to nucleotides 76–95 and 96–115 of CV-IIIb (Rakowski et al., 1994). CVd-IV primers are complementary and homologous to nucleotides 199–216 and 217–235 of CVd-IV (Puchta et al., 1991).

synthesised by a transcription reaction with T7-RNA-polymerase in the presence of DIG-labelled UTP. Full-length DIG-labelled monomeric DNA probes were synthesised by PCR amplification in 50 µl reaction volume containing 0.5 µM of each primer (Table 1), 1.5 mM $MgCl_2$, 120 µM each of the four dNTPs (containing DIG-labelled dUTP) and 1 U of *Taq* DNA polymerase and 1.5–2 ng of the template. Reactions consisted of a denaturation step of 5 min at 95 °C, 35 cycles (10 s at 92 °C, 10 s at 50 °C and 20 s at 72 °C) and a final extension step of 5 min at 72 °C. PCR products were analysed in 2% agarose gels to verify the presence of a band of the expected size. DNA concentration was measured with the spectophotometer and the amount of DIG-labelled DNA estimated against commercial positive controls.

Prehybridisation and hybridisation were carried out in 50% formamide and 6× SSPE as described by Maniatis et al. (1989). The membranes were prehybridised at 42 °C for 2–4 h and hybridised overnight at 65 °C (RNA-probes) or 50 °C (DNA-probes). After hybridisation, they were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min, followed by another wash in 0.1× SSC, 0.1% SDS for 60 min at 68 °C (RNA-probes) or 60 °C (DNA-probes). The DIG-labelled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualised with the chromogenic substrate 4-nitro blue tetrazolium chloride (NBT) and X-phosphatase or the chemiluminescence substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) (Boehringer Mannheim).

When imprinted membranes were hybridised against DIG-labelled RNA-probes and DNA-probes of each viroid, positive reactions were consistently obtained only when the CSPD substrate was used for detection. As reported earlier (Romero-Durbán et al., 1995), chromogenic detection was not sufficiently sensitive for the detection of citrus viroids. Imprints made from longitudinal and transverse stem segments either from young succulent tissues or from older tissues were all adequate, but older tissues were more easily imprinted and gave a larger spot which facilitated the identification of positive hybridisation signals.

CVd-I and CVd-III probes produced background hybridisation signals which were consistently avoided when the membranes were soaked in 2 M mercaptoethanol and rinsed with water before prehybridisation. Under these conditions, all the probes were highly specific, except the CEVd RNA-probe which also showed a weak hybridisation signal against CVd-IV. Cross-hybridisation between CEVd and CVd-IV had already been reported (Duran-Vila et al., 1988) and is due to the sequence homology found between both viroids (Puchta et al., 1991). Conversely the lower affinity of DNA probes with the viroid template resulted in a weaker or even imperceptible cross-hybridisation signal, but in all instances the hybridisation between each viroid and the homologous probe was satisfactory.

Mixtures of the five RNA-probes or the five DNA-probes were assayed against membranes imprinted with citrons previously inoculated with field isolates which contained several viroids (Figure 1A, above) or single viroids sources (Figure 1A, below). Probe mixtures were able to discriminate between viroid-free and viroid-infected tissues (Figure 1B,C). The intensity of the hybridisation signals did not correlate with the concentrations of specific viroids observed by sPAGE analysis (Figures 1 and 2). This observation may reflect differences in the secondary structures that viroids may acquire *in vivo*, thus resulting in different degrees of accessibility for binding with the probes.

DNA-probes were cheaper to produce than RNA-probes and could be handled without the precautions needed to avoid the degradation of single stranded RNA. DNA-probes have been successfully frozen, stored and thawed several times and never presented problems associated with poor handling of RNA-probes. To establish the conditions for sensitive and cost efficient indexing tests, assays were performed to estimate the amount of probe to be used in each hybridisation assay. The use of 20 ng of each DNA-probe (which contained 240 pg of DIG-labelled DNA) per cm^2 of membrane was suitable and the hybridisation signal was not enhanced by increasing the amount of probe. Under these conditions, a single hybridisation using a mixture of five DNA-probes provided an ideal system to perform routine viroid indexing in a single hybridisation assay. Alternatively, the viroids present in a given isolate could be identified by hybridisation against single viroid probes (Figure 2). Mixtures of five DNA-probes and single viroid probes gave definitive signals when tested against the collection of citrus viroids available at IVIA (data not shown).

Assays were also conducted to evaluate the sensitivity of this method to detect viroids from commercial species and cultivars growing in the field. As also revealed by analysis of nucleic acid preparations by sPAGE and dot-blot hybridisation, the detection

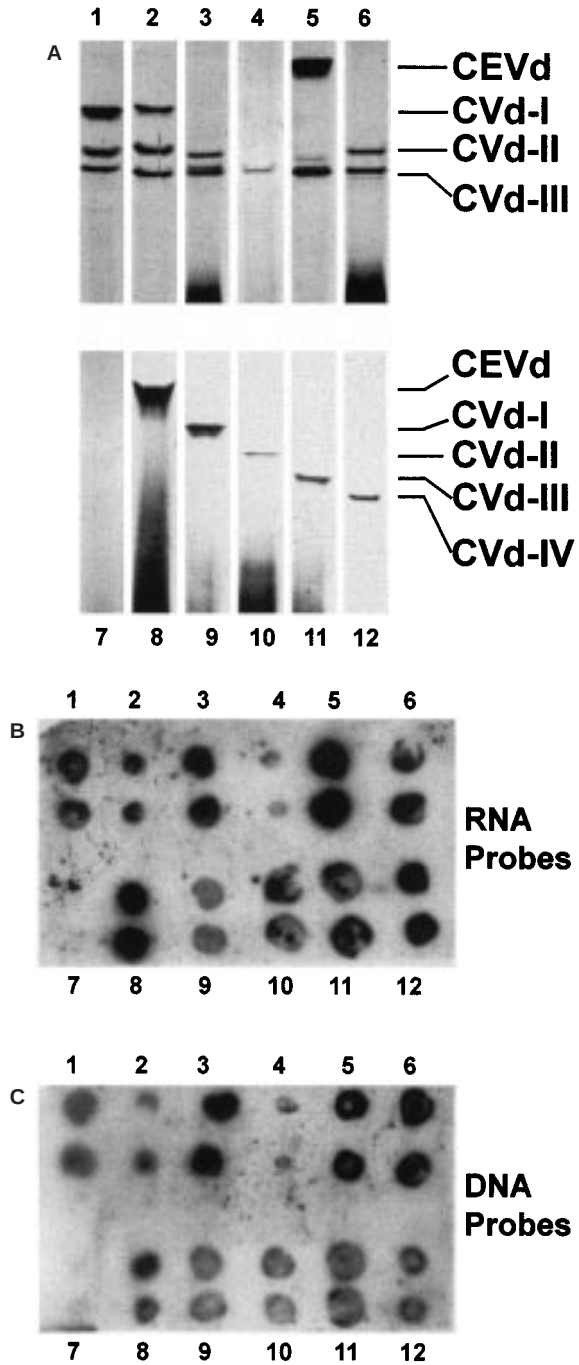

Figure 1. Imprint hybridisation against citrons inoculated with field isolates and single viroid sources using mixtures of five RNA- or DNA-probes. Samples from citrons inoculated with field isolates containing several viroids (A above: lanes 1–6), uninoculated control (A below: lane 7) and single viroid sources (A below: lanes 8–12) as revealed by sPAGE and silver staining, were imprinted and hybridised against a mixture of RNA-probes (B) or DNA-probes (C). For each sample, imprints were made with two transverse stem segments.

of citrus viroids from species other than citron, was erratic (Palacio, unpublished results). Some of the factors which affect the detection of citrus viroids from commercial trees known to be infected are: (a) long incubation periods, ranging from a few months to several years since inoculation until the viroids reach detectable concentrations; (b) differences in detectable amounts among species and cultivars; (c) seasonal differences which may vary considerably from one year to the next. Therefore, the use of citron as an amplification host is critical to achieve the highest sensitivity.

In order to establish the minimum incubation period necessary for viroid detection from inoculated citrons and the effect of the incubation temperature, two sets of plants were graft inoculated with the same viroid sources described above and a cachexia variant (CVd-IIb) of HSVd. The plants were kept at 18–25 °C until growth of the scion bud was observed in at least half of the plants of each lot. One set of plants was then transferred to a warm greenhouse set at 28–32 °C, whereas the other set was kept at 18–25 °C. The plants kept under the two temperature regimes were sampled at monthly intervals. For each inoculation and incubation treatment, the samples were collected from two plants and processed separately. The results (Table 2) showed positive detection regardless of the incubation temperature. Minimum incubation periods for detection of all citrus viroids ranged from three months when the inoculated citrons were kept at 28–32 °C to seven months when the plants were kept at lower temperatures. These results indicate that imprint hybridisation is sensitive enough to detect the viroids even from citrons grown at temperatures below those considered as optimal for viroid replication/accumulation and symptom expression.

This method has been adopted as the indexing procedure for the certification programme conducted at the Instituto Valenciano de Investigaciones Agrarias. With a single hybridisation assay using a mixture of the five probes viroid-free and viroid-containing tissues can be easily discriminated. Alternatively, with hybridisation against viroid specific probes, the viroids present in a viroid infected source can be identified. The extensive application of this protocol during the last two years demonstrated that the procedure is as sensitive and reliable as sPAGE analysis. In addition,

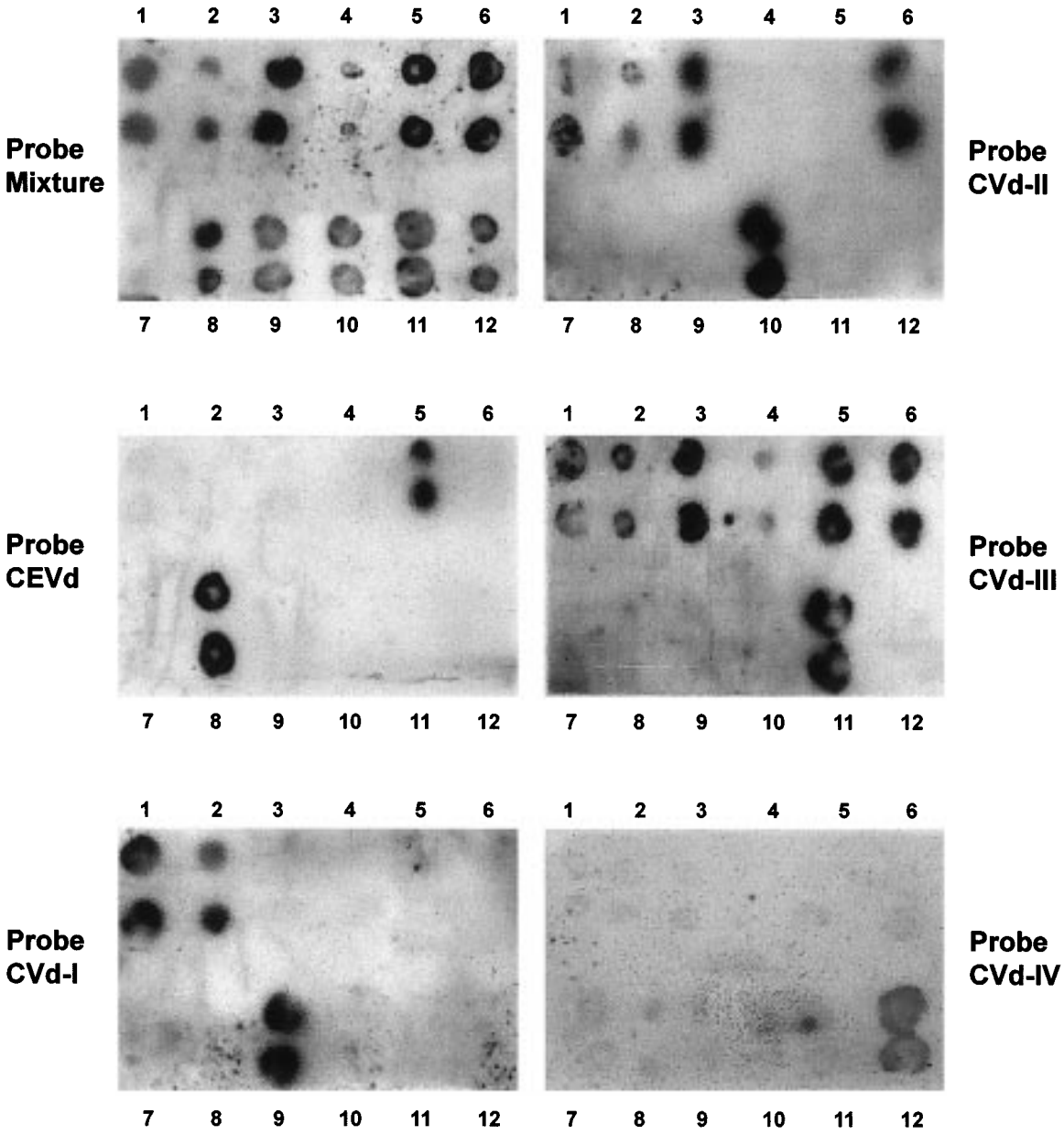

Figure 2. Imprint hybridisation against samples of citron plants inoculated with field isolates (samples 1–6), uninoculated control (7) and single viroid sources (8–12) using a mixture of the five DNA-probes and single viroid probes. Source of tissue and membrane design were identical to those shown in Figure 1.

once probes are available, the quick analysis of large numbers of samples can be readily performed by easily trained personnel with minimal sample manipulation and inexpensive equipment. With the availability of cloned plasmid DNA containing full-length sequences of the five citrus viroids, DNA-probes can be easily synthesised, and therefore may be produced and commercialised as detection kits. Once commercial kits are available, indexing tests could be performed in national indexing laboratories of citrus growing countries with

Table 2. Detection of citrus viroids on inoculated citrons: effect of incubation period and temperature[1]

Viroid	Incubation period (months)							
	28–32 °C			18–25 °C				
	1	2	3	1	2	3	5	7
CEVd	++	++	++	++	++	++	++	++
CVd-Ia	++	++	++	++	++	++	++	++
CVd-IIa	−−	−+	++	−−	−−	−+	−+	++
CVd-IIb	−−	++	++	−−	−−	−+	−+	++
CVd-IIId	++	++	++	−+	++	++	++	++
CVd-IV	++	++	++	−−	−−	−+	−+	++

[1] For each treatment and incubation period two plants were analysed (two tissue imprints each) separately: ++ = positive detection in both plants; +− = positive detection in only one of the plants; −− = no detection in either plant.

limited availability of equipment or in specialised nurseries that wish to perform their own tests.

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