Indexing of Citrus Viroids by Imprint Hybridization: Comparison with Other Detection Methods

A. Palacio, X. Foissac, and N. Duran-Vila

ABSTRACT. An imprint hybridization method has been designed to detect viroids from infected tissues. Freshly cut tissue pieces from inoculated citrons can be imprinted by firmly pressing the cut surface onto positively charged Nylon membranes. Imprints, once fixed on the membrane, can be processed immediately or stored. Processing of the imprinted membranes involves: a) availability of probes; b) hybridization of the membranes against the DIG-labeled viroid probes; c) detection of DIG-labeled hybrids (using an anti-DIG-alkaline phosphatase conjugate); d) visualization of the DIG-alkaline phosphatase (using the chemiluminiscent substrate CSPD). With a single hybridization using a mixture of the five probes, viroid-free, and viroid-containing samples can be easily discriminated. Alternatively, with hybridization against viroid specific probes, the viroids present in a viroid-containing source can be identified. Nucleic acid analysis of inoculated citrons by imprint hybridization provides a fast and reliable method for routine indexing of citrus viroids and it has advantages in terms of sensitivity and cost over other indexing procedures.

Index words. Exocortis, cachexia, sPAGE.

Citrus harbors a complex of citrus viroids which are widespread in old cultivars in all citrus-growing countries. At least two viroids, CEVd and specific variants of CVd-II (HSVd), are causal agents of the exocortis and cachexia diseases, respectively. It has been demonstrated that other viroids may also cause symptoms and/or different degrees of stunting (8, 19, 23). Indirect evidences suggest that other diseases (gummy bark, gum pocket-gummy pitting and Kassala disease) may be caused by viroids (2, 15, 16, 21).

Viroid control is critical for the commercial propagation of budwood released from quarantine, sanitation and certification programs. These programs require the performance of large numbers of indexing tests which must be sensitive, reliable, and as quick and economical as possible.

In the past, viroid detection has been performed by biological indexing on indicator hosts. Nucleic acid analysis technologies have been proposed as an alternative or as a complementary tool to conventional biological indexing (5, 9, 11, 12, 13, 24, 25) of citrus viroids. Here we report the attempts to improve and/or simplify current viroid indexing methods by a tissue imprinting hybridization protocol.

MATERIALS AND METHODS

Source of tissue for viroid detection from field grown plants. Viroid infected trees growing in experimental plots of the Instituto Valenciano de Investigaciones Agrarias (IVIA) were selected to evaluate different detection methods on commercial species growing in the field. The trees were commercial cultivars (Navelina sweet orange, Washington navel sweet orange, Nules clementine, and Verna lemon) grafted on Carrizo citrange, Navelate sweet orange grafted on trifoliate orange and seedlings of Orlando tangelo, Alemow and Parson's Special mandarin. All the trees had been graft-inoculated before transplanting to the field with the complex viroid isolate E-117, which had been characterized by sPAGE and molecular hybridization, as containing four different viroids (CEVd, CVd-I,

CVd-II, CVd-III) (Palacio et. al., unpublished data). At least two trees of each species and cultivar were sampled three times year (August-September, November-December, April-May) during a 3-yr period. Tissue samples consisted of either, young leaves and stems or bark. The same samples were subjected to nucleic acid analysis by sPAGE and slot-blot hybridization.

Source of tissue for development of imprint hybridization techniques. The selection 861-S1 of Etrog citron grafted onto rough lemon rootstock was used as a source of tissue. The plants were graft-inoculated from several viroid-infected sources maintained at the viroid collection of IVIA. The viroid sources were: CEVd (variant CEVd-117) (7), CVd-I (Foissac and Duran-Vila, unpublished data), HSVd (variant CVd-IIa-117) (17) CVd-III (Foissac and Duran-Vila, unpublished data) and CVd-IV (kindly provided by J. S. Semancik, Univ. Calif., Riverside) (6, 21). Inoculated plants and uninoculated controls were kept in a greenhouse at 28° to 32°C and were indexed by sPAGE nucleic acid analysis (5, 6, 21) before use as a source for tissue imprinting.

In order to define the incubation period necessary for sensitive detection by imprint hybridization, two sets of plants of the selection 861-S1 of Etrog citron grafted on rough lemon rootstock, containing seven lots of two plants each, were graft inoculated with the same viroid sources described above. In addition, two cachexia-inducing sources of HSVd (X-704 and X-707) (17) were also included. Graft inoculation on the rough lemon rootstock and propagation of the citron were done simultaneously in October. The plants were kept at 18° to 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° to 32°C, whereas the other set was kept at 18° to 25°C. Plants from both sets of plants were sampled at monthly intervals. For each inoculation and incubation treatment, the samples were collected from two plants and processed separately. For comparison purposes, the same samples were subjected to sPAGE analysis, slot-blot hybridization and imprint hybridization.

Nucleic acid extraction and analysis. Samples (5 g) of tissue were homogenized in 5 ml volume of extraction medium (0.4M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) mercaptoethanol) and 15 ml of water saturated phenol (22). The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10mM Tris-HCl; 10mM KCl; 0.1mM $MgCl_2$; pH 7.4).

Aliquots (20 µl) equivalent to 300mg of fresh weight tissue were analyzed by sPAGE and the viroid bands were viewed by silver staining (6). Aliquots (10 ml) of the same samples were pretreated in 6×SSC and 8% formaldehyde for 15 min at 60°C and blotted onto positively charged Nylon membranes (Boehringer Mannheim®) using an Hybri-slot filtration manifold (BRL®), immobilized by UV crosslinking and hybridized against DIG-labeled viroid probes.

Preparation of imprinted membranes. Stems were freshly cut longitudinally and/or transversely and firmly pressed onto the surface of positively charged Nylon membranes (20). The imprinted samples were immobilized by UV cross-linking and were stored in the dark until processing. To avoid background hybridization signals, the imprinted membranes were pretreated in 2M mercaptoethanol for 10 min and rinsed twice with water before prehybridization.

Synthesis of digoxigenin (DIG)-labeled probes. Digoxigenin

(DIG)-labeled DNA probes were obtained using a cloned plasmid containing full-length viroid monomeric DNA. Viroid DNA was synthesized by retrotranscription and PCR amplification from the LiCl-soluble fraction essentially as described by Yang et al. (25) using synthetic oligonucleotides complementary and homologous to the sequence of the upper strand of the C region of each viroid (Table 1). The amplified DNA was ligated to *Eco* R-V restricted pT7-Blue vector (Novagen®) and the inserts were sequenced with the ABI PRISM DNA sequencer 377 (Perkin-Elmer®).

DIG-labeled DNA probes were synthesized by PCR amplification of cloned sequences 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-labeled dUTP) and 1 U of Taq DNA polymerase. Reactions consisted of a denaturation step of 5 min at 95°C, 35 cycles (10 s at 92°C, 10s at 50°C and 20s at 72°C) and a final extension step of 5 min at 72°C. PCR products were analyzed in 2% agarose gels to verify the presence of a band of the expected size. DNA concentration was measured with the spectrophotometer and the amount of DIG-labeled DNA estimated against commercial positive controls.

Hybridization and detection. Prehybridization and hybridization were carried out in 50% formamide and 6×SSPE as described by Maniatis et al. (14). The membranes were prehybridized at 42°C for 2 to 4 h and hybridized overnight at 50°C. After hybridization, 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 60°C. The DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualized with the chemiluminescent substrate CSPD (Boehringer Mannheim®).

RESULTS

Viroid detection from field grown plants. The suitability of sPAGE and slot-blot hybridization for viroid detection from commercial species growing in the field was evaluated. As reported earlier (3, 12), bark tissue yielded higher viroid titers than young succulent leaves and higher viroid titers were recovered from summer and/or fall samplings. However, the results of a 3-yr period indicated that the indexing results were unreliable. All the viroids were detected in analysis performed on Navelate sweet orange, Nules clementine, Verna lemon and

TABLE 1
COMPLEMENTARY AND HOMOLOGOUS OLIGODEOXYRIBONUCLEOTIDE PRIMERS FOR CITRUS VIROIDS

Viroid	Primers[z]	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[z]	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'

[z]Described by Astruc et al. (1) and kindly provided by Dr. V. Pallás and Dr. J. F. Marcos (CEBAS, CSIC, Murcia, Spain).

alemow tissues. However, as indicated earlier for Navelina sweet orange and grapefruit (4, 10), CEVd and CVd-I detection failed or was erratic when samples of Washington Navel, Palestine sweet lime, Orlando tangelo, and Parson's Special mandarin were analyzed (Fig. 1 A). Surprisingly, samples of trees of Verna lemon which indexed positive for the four viroids over a 2-yr period, gave negative results when analyzed the following year (Fig. 1B).

These results indicate that, under our growing conditions, direct indexing of field grown trees is unreliable and cannot be used as a routine detection method in certification programs. Whereas positive indexing results are always conclusive, the results of a 3-yr study indicate that negative results can be obtained from plants known to be infected. Uneven distribution of the viroids within the hosts, seasonal variations and year-to-year fluctuations in titer may account for these unexpected results.

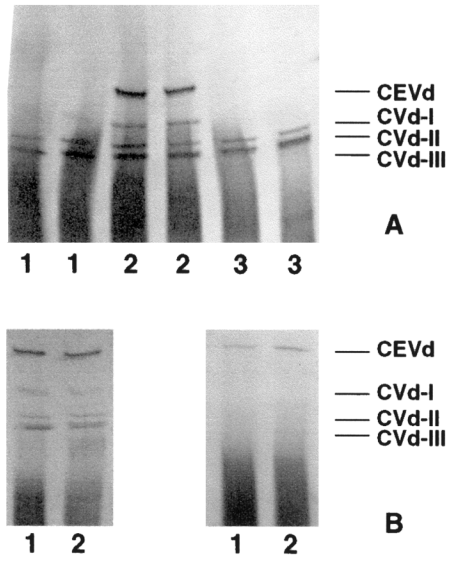

Fig. 1. sPAGE nucleic acid analysis of different citrus species inoculated with the citrus viroids isolate E-117. A) Analysis of samples of Palestine sweet lime (1), Alemow (2) and Orlando tangelo (3). B) Positive detection of citrus viroids in samples collected from two separate Verna lemon trees (1, 2) (left) compared with the results obtained when the same two trees (1, 2) were analyzed the following year (right).

Imprint hybridization from inoculated citrons. In order to simplify the viroid detection procedure using the citron indicator as an amplification host, an imprint hybridization protocol was assayed. When imprinted membranes were hybridized against DIG-labeled probes of each viroid, positive reactions were consistently obtained against viroid infected samples. 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 hybridization signals. The use of 20 ng of each DNA-probe (which contained 240 pg of DIG-labeled DNA) per cm^2 of membrane was suitable and the hybridization signal was not enhanced by increasing the amount of probe.

The hybridization results obtained using a mixture of the five probes were satisfactory to discriminate viroid-free from viroid-infected tissues (Fig. 2). Hybridization analysis using single specific probes allowed the identification of the viroids present in isolates containing mixtures of several viroids (Fig. 3). The intensity of the hybridization signals did not correlate with the titers of specific viroids observed by sPAGE analysis (Fig. 3). 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. The results of sPAGE and imprint hybridization analysis of a complex isolate (Fig. 3, lane 5) suggests that the two fast migrating bands correspond to two different variants of CVd-III which appear to

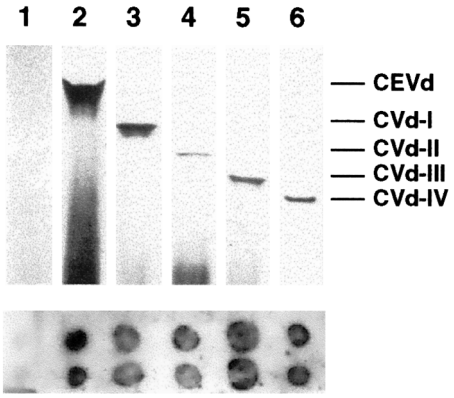

Fig. 2. Analysis of citrons infected with single viroid sources by sPAGE (above) and imprint hybridization using a mixture of five specific viroid probes (below).

accumulate in different titers. The existence of CVd-III sources present in low titers in inoculated citrons, although unusual, is illustrated by the low concentration found in one of the isolates analyzed (Fig. 3, lane 4).

The imprint hybridization protocol has been tested against the collection of citrus viroids available at IVIA with satisfactory results (data not shown).

Determination of minimum incubation periods for sensitive detection on inoculated citrons. An assay was conducted to: a) determinate the minimum incubation period required for viroid detection; b) assess the effect of incubation temperature; and c) compare the sensitivity of the imprint hybridization protocol with sPAGE and slot-blot hybridization analysis. The results indicate that imprint hybridization is sensitive for viroid detection even from citrons grown at temperatures below those considered as optimal for viroid replication/accumulation (Table 2). The sensitivity and incubation periods required were similar to those required for sPAGE and slot-blot hybridization analysis.

Detection of CVd-IV by imprint hybridization appears to be especially dependent of the incubation temperature. Whereas the viroid was detected after one month in plants incubated at 28° to 32°C, two to six additional months were required for its detection at 18° to 25°C (Table 2). The discrepancy between detection by imprint hybridization and either sPAGE or slot-blot hybridization analysis can only be explained by temperature dependent changes in the secondary structure of CVd-IV *in vivo*, resulting in a more accessible conformation for binding with the probe at 28° to 32°C.

This imprint hybridization procedure has been adopted as the index-

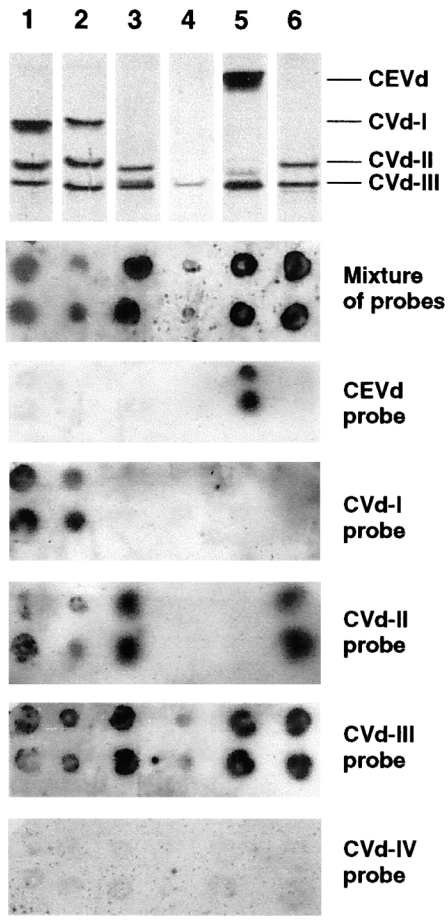

Fig. 3. Analysis of citrons infected with field isolates containing several viroids by sPAGE and imprint hybridization.

TABLE 2
DETECTION OF CITRUS VIROIDS ON ARIZONA ETROG CITRON: EFFECT OF INCUBATION TEMPERATURE[z]

	28° to 32°C			18° to 25°C				
	Incubation period (mo)			Incubation period (mo)				
	1	2	3	1	2	3	5	7
Viroid	sPAGE			sPAGE				
CEVd	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-I	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-IIa	− −	− +	+ +	− −	− −	− −	+ +	+ +
X-704	− −	+ +	+ +	− −	− −	− −	+ +	+ +
X-707	− −	− +	+ +	− −	− −	− −	− +	+ +
CVd-III	+ +	+ +	+ +	− +	− +	+ +	+ +	+ +
CVd-IV	− −	− +	+ +	− −	− +	+ +	+ +	+ +
	Slot-blot hybridization			Slot-blot hybridization				
CEVd	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-I	+ +	+ +	+ +	− +	+ +	+ +	+ +	+ +
CVd-IIa	− −	+ +	+ +	− −	− −	− −	+ +	+ +
X-704	− +	+ +	+ +	− −	− −	− −	+ +	+ +
X-707	− −	+ +	+ +	− −	− −	− −	− +	+ +
CVd-III	++	+ +	+ +	− +	− +	+ +	+ +	+ +
CVd-IV	− −	− +	+ +	− −	− −	+ +	+ +	+ +
	Imprint hybridization			Imprint hybridization				
CEVd	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-I	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-IIa	− −	− +	+ +	− −	− −	− +	− +	+ +
X-704	− −	+ +	+ +	− −	− −	− +	− +	+ +
X-707	− −	+ +	+ +	− −	− +	+ +	+ +	+ +
CVd-III	+ +	+ +	+ +	− +	+ +	+ +	+ +	+ +
CVd-IV	+ +	+ +	+ +	− −	− −	− +	− +	+ +

[z]For each treatment and incubation period, two plants were analyzed separately; + + = positive detection in both plants; + - = positive detection in only one of the plants; - - = no detection in either plant.

ing method for the certification program conducted at the Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain. The extensive application of this protocol during the past two years demonstrated that the procedure is as sensitive and reliable as sPAGE analysis.

DISCUSSION

Improved detection procedures must be superior to other available methods in terms of sensitivity, reproducibility, reliability, and cost. Attempts to detect viroids from different species and cultivars growing in the field gave unreliable results. In earlier work, the suitability of nucleic acid sPAGE analysis from inoculated citrons was shown to provide superior results over conventional biological indexing in terms of sensitivity and cost. In addition, the incubation temperature of the inoculated citrons were less critical than that required for symptom expression, and the period necessary for detection of all the viroids tested including the cachexia agent could be shortened considerably (5). Although nucleic acid and sPAGE analysis improved sensitivity and reduced costs for

viroid detection, the laboratory manipulations associated with the molecular analysis have been the limiting factor in terms of the number of indexing tests to be performed at a given time.

Viroids can also be detected by molecular hybridization of imprinted membranes (18, 20), avoiding the need to process the samples. The sensitivity achieved using DIG-labeled RNA probes and the NBT substrate for anti-DIG-alkaline phosphatase conjugate was insufficient to detect all citrus viroids (20), but the enhancement achieved with the chemiluminescent substrate CSPD resulted in adequate detection (Palacio, unpublished results). In addition, DIG-labeled DNA probes which are cheap to produce, stable upon storage, and easy to handle can be used with satisfactory sensitivity.

With a single hybridization assay using a mixture of the five probes, viroid-free and viroid-containing tissues can be easily discriminated. Alternatively, with hybridization against single viroid specific probes, the viroids present in a viroid infected source can be identified. The specificity of the method allows detection of distinct citrus viroids at the species level but not sequence variants within a species. Therefore, in order to differentiate cachexia inducing variants within CVd-II, additional tests by biological indexing or molecular hybridization against specific oligonucleotide probes (13) are required.

The method is easy to handle and its sensitivity is retained even when the inoculated citrons are kept under suboptimal conditions provided that the incubation period is sufficient. The suitability of the incubation period must be verified by including proper positive controls.

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