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Author

Flores, R.

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Detection of Citrus Exocortis Viroid in Natural and Experimental Citrus Hosts by Biochemical Methods

R. Flores

ABSTRACT. Citrus exocortis viroid (CEV) was detected in extracts of mature leaves collected in winter from field trees by double polyacrylamide gel electrophoresis and silver staining. Nucleic acid preparations were obtained by phenol extraction and further fractionation with cellulose chromatography and LiCl. A band comigrating with the circular molecules of CEV was detected in the 9 trees indexed as CEV-infected including lemon, sweet orange and clementine mandarin samples. In the case of one of the clementine samples some bands were particularly visible that probably correspond to other smaller RNAs which have been associated with the exocortis syndrome. As an alternative technique, CEV was detected in nucleic acid extracts from Etrog citron by dot-blot hybridization. Preparations of either total nucleic acids or of those soluble in 2M LiCl were denatured with formaldehyde and applied to nitrocellulose membranes that were probed with a CEV-cDNA. A high background was observed with the healthy controls which could be very much reduced by incorporating the fraction of nucleic acids insoluble in 2M LiCl into the hybridization solution. Whether a similar approach can be used with field trees where CEV is present at much lower concentrations remains to be determined.

Index words. Double polyacrylamide gel electrophoresis, dot-blot assays.

Detection of virus diseases by biochemical techniques of the type of enzyme-linked immunosorbent assays (ELISA) is a rapid and economical approach. Nevertheless, this technology cannot be applied to citrus exocortis, since this disease is induced by citrus exocortis viroid (CEV) (13), and viroids are circular single-stranded RNAs which are not encapsidated by a coat protein (3), the antigenic component of viruses. Two other alternative biochemical methods have been proposed for the detection of maladies caused by viroids: double polyacrylamide gel electrophoresis (11) and dot-blot hybridization (9) based, respectively, on the specific circular structure and the nucleic acid sequence in viroids. The aim of this paper is to present the results of the application of both techniques to the detection of CEV in natural and experimental citrus hosts, and to discuss their use in the diagnosis of this viroid.

MATERIALS AND METHODS

Extraction and fractionation of nucleic acids from field trees. Samples of mature leaves (100 g) of different origins, collected in winter, were extracted with buffer-saturated

phenol as indicated previously (12). The aqueous phases were adjusted with HCl to pH 7.0, brought to a final volume of 200 ml with distilled water, and prepared so as to contain STE buffer (50 mM Tris-HCl pH 7.0, 100 mM NaCl and 1 mM EDTA) and 35% ethanol. After adding 3 g of cellulose powder (CF-11 Whatman) (7), the mixtures were left at 4 C with moderate stirring for variable times (from 1 h to overnight). The cellulose pellets obtained by centrifugation at 1000 g for 5 min, were washed five times in centrifuge tubes with 50 ml of 25% ethanol in STE. The nucleic acids were released from the cellulose with a final wash of 20 ml of STE and precipitated with 2.5 volumes of ethanol at -30 C for 2 h. The pellets collected by centrifugation at 8000 g for 20 min were dried, resuspended in 0.4 ml of STE and, following the addition of 0.1 ml of 10M LiCl, were left overnight in an ice water bath. The nucleic acids remaining in the supernatant after centrifuging at 8000 g for 20 min, were recovered by ethanol precipitation as indicated above, dried and resuspended in 0.25 ml of TKM buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl, 0.1 mM MgCl₂).

Analysis by double polyacrylamide gel electrophoresis (PAGE). For this purpose the method of Schumacher *et al.* (11) was followed with the modifications reported previously (6). Nucleic acid preparations (equivalent to 30 g of initial tissue) were electrophoresed in a non-denaturing gel that was stained with ethidium bromide. The section containing the CEV position (fig. 1A) was placed on top of a denaturing gel, electrophoresed and stained with silver as reported by Igloi (8).

Synthesis of a complementary DNA probe to CEV. Purification of CEV and synthesis of a complementary DNA (CEV-cDNA) by the random primer method (14), was carried out as previously published (5).

Preparation of samples for dot-blot hybridization. Preparations of nucleic acids from healthy and CEV-infected Etrog citron were obtained by extracting young foliar tissue with buffer-saturated phenol as indicated above, followed by precipitation with ethanol either directly (total nucleic acids) or after fractionation with 2M LiCl (LiCl soluble nucleic acids), and resuspension in TKM buffer (final volume of 1 ml starting from 1 g of tissue). Samples were denatured with formaldehyde (16) and applied to nitrocellulose membranes (15), that were prehybridized and hybridized as reported previously (5). Calf thymus DNA in the hybridization solution was replaced with a preparation of nucleic acids insoluble in 2M LiCl from healthy plants (5) where indicated.

RESULTS

Detection of CEV from field trees by double PAGE. Fig. 1A shows that a band comigrating with CEV was observed by ethidium bromide staining in some of the samples in the non-denaturing PAGE. This pattern became much more clear after the second denaturing PAGE and silver staining, where a band comigrating with the apparent circu-

lar molecules of CEV (CEV_c) was observed in all the samples studied from trees indexed by bioassay as CEV-infected (fig. 1B). The type and number (in brackets) of the trees analyzed were: Verna lemon [2], Fino lemon [2], Navel-late sweet orange [1], Nules clementine [1], Hernandina clementine [1], and Oroval clementine [2]. The low intensity of the CEV_c band in the case of sweet orange Navel-late is probably a reflection of the poor nucleic acid recovery that was obtained with this extract. This band was absent in a healthy control of Verna lemon. In the sample of Nules clementine, other bands below CEV_c were particularly visible. These probably correspond to some of the RNAs smaller than CEV which also have been associated with the exocortis syndrome (4, 10).

Detection of CEV from citron by dot-blot hybridization. Fig. 2A and C shows that a high background was observed in the healthy controls (especially in the case of total nucleic acids). This nonspecific binding of the probe could be very much reduced, as previously reported in *Gynura aurantiaca* and *Chrysanthemum morifolium* (5), by incorporating into the hybridization mixture the fraction of nucleic acids from healthy plants insoluble in 2M LiCl (fig. 2B and D). The sensitivity of the method has been estimated in 50-100 pg of CEV per spot (5), allowing easy detection of viroid in amounts equivalent to those found in 0.1-0.2 mg of fresh tissue.

DISCUSSION

Detection of CEV in citrus samples has been reported previously using either non-denaturing PAGE and ethidium bromide staining (1), or two consecutive steps of non-denaturing and denaturing PAGE and silver staining (2). In both cases reproducible results were obtained with glasshouse-grown citron leaves, whereas with field trees consistent results were observed only when using young

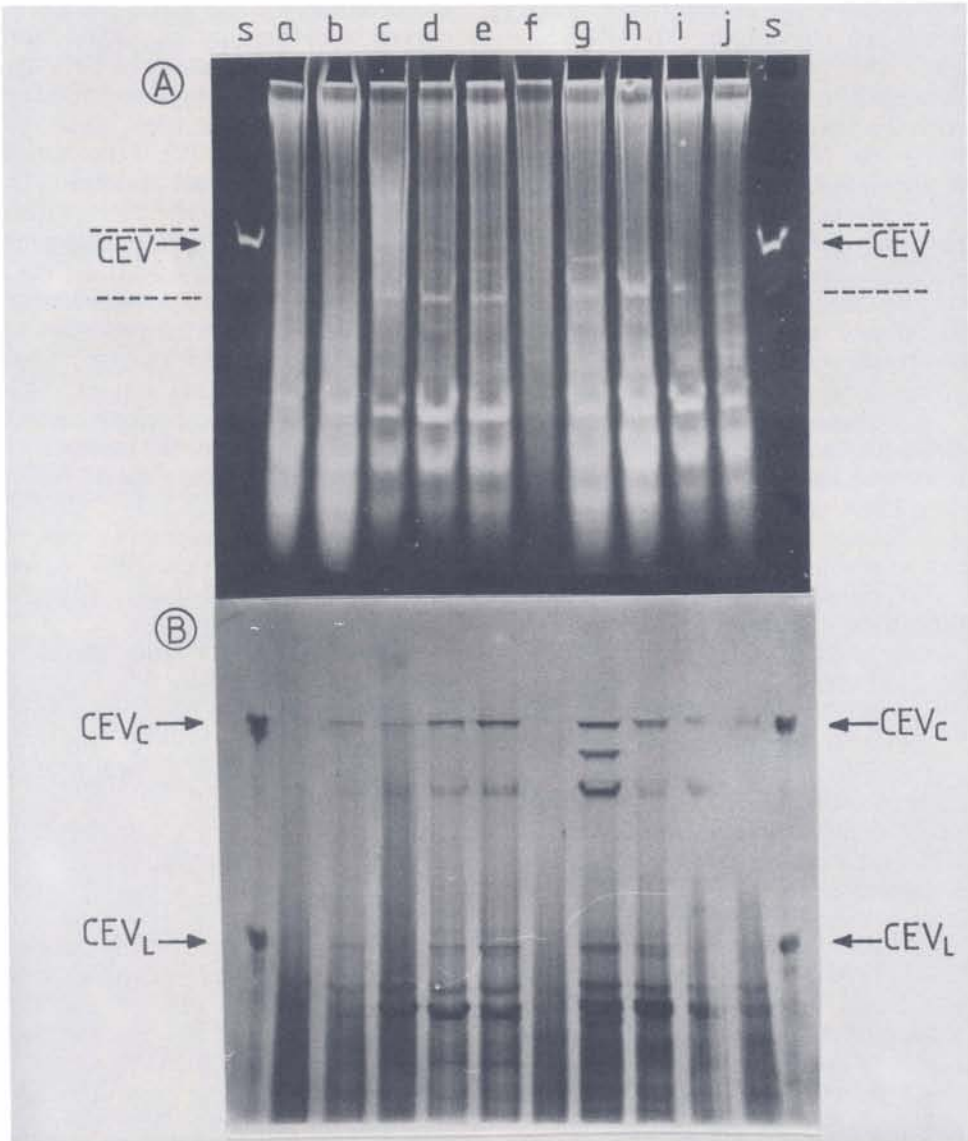


Fig. 1. Polyacrylamide gel electrophoresis under nondenaturing (A) and denaturing conditions (B) of nucleic acid preparations from citrus field trees. Lanes *s*, standard purified CEV-RNA whose position is indicated by arrows. Lane *a*, extract from a healthy control of Verna lemon. Lanes from *b* to *j* extracts from CEV-infected trees. Verna lemon (*b* and *c*), Fino lemon (*d* and *e*), Navel-late sweet orange (*f*), Nules clementine (*g*), Hernandina clementine (*h*), and Oroval clementine (*i* and *j*). The non-denaturing gel (A) was stained with ethidium bromide and the area indicated within dashed lines was cut and applied on top of a denaturing gel (B) that was stained with silver. In this last case two bands corresponding to the circular and linear CEV forms (CEV_c and CEV_l) can be observed.

leaf samples from sweet orange and grapefruit collected in summer (1), or from a heavily pruned Tarocco sweet orange tree collected in spring-summer (2). With the method described here, it was possible to detect CEV in the nine field trees examined even

from samples of mature leaves collected in winter. Several points should be emphasized. Since CEV is present in its natural hosts at very low concentrations, it is necessary to purify the viroid from large amounts of tissue and yet not overload the

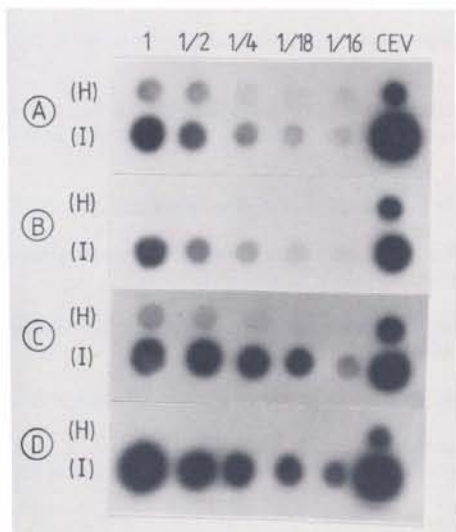


Fig. 2. Autoradiograph of a spot hybridization of preparations of total nucleic acids (A and B) or 2M LiCl soluble nucleic acids (C and D) of healthy (H) and CEV-infected (I) citron leaf tissue probed with CEV-cDNA. The hybridization mixture contained 250 μ g/ml of calf thymus DNA (A and C) or the fraction of nucleic acids from healthy plants insoluble in 2M LiCl (B and D). The spots on the last column on the right correspond to 0.5 ng (upper) and 5 ng (lower) of purified CEV.

gels. Cellulose chromatography was a very powerful approach for removing a great deal of contaminants. In cases where a precipitate was observed when the aqueous phase from the initial extraction was made up to 35% ethanol, the concentration could be reduced to 25% without affecting the results. The double PAGE could be applied after this step, although no clear bands were visible after the first non-denaturing cycle (data not shown), probably due to the presence of polysaccharides. The final fractionation with LiCl clarified this pattern

and allowed the observation of the bands corresponding to CEV and cellular RNAs in some cases. In addition, smaller RNAs other than CEV that have been associated with the exocortis syndrome (4, 10) could be observed in some of the samples.

Detection of CEV from citron by dot-blot hybridization presented similar problems to those reported previously with the herbaceous hosts of CEV (5). The approach used to circumvent the nonspecific binding to the probe with herbaceous hosts was also valid with citron, but has not been tested with field trees. Since the concentration of CEV is much lower, extracts corresponding to higher amounts of initial tissue should be applied to the membranes and therefore, more intense nonspecific background can be expected. Alternatively, the extracts could be more extensively purified, but this reduces the usefulness of the technique as a rapid indexing method. Finally, if detection of the RNAs smaller than CEV is also desired, the corresponding cDNAs for these should be also included in the hybridization solution since they do not cross-hybridize with CEV (4). The possibility of substituting radioactive for chemically labeled probes, will certainly stimulate studies to overcome the present problems and permit use of this technique for the rapid diagnosis of exocortis.

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