Diagnosis of Specific Viral RNA Sequences in Plant Extracts by Hybridization with a Polynucleotide Kinase-Mediated, ^{32}P-Labeled, Double-Stranded RNA Probe

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ABSTRACT

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A method was developed for screening of virus-related RNAs in plant extracts. The method is based on the hybridization of a polynucleotide kinase-mediated, ^{32}P-labeled, double-stranded (ds) RNA probe with RNA or sap extracts. The cucumber mosaic virus (CMV) and associated RNA-5(CARNA-5) was used as a model system. Naturally infected plants of *Nicotiana glauca* were screened for the presence of dsCARNA-5; dsRNA was purified on CF-11 columns and analyzed in polyacrylamide gels. Three types of low-molecular-weight CARNA-5-like dsRNA species, with estimated molecular weights of (type I) 0.27, (type II) 0.22, and (type III) 0.19×10^6 daltons, were observed in plants of *N. glauca* that reacted with CMV antiserum. Types II and III were shown to contain CARNA-5 sequences by Northern blot hybridization of dsRNA patterns with ^{32}P-labeled dsCARNA-5. This labeled probe was further used to detect CARNA-5 infection in *N. glauca* and *N. glutinosa* plants by hybridization with dot-spots of dsRNA as well as plant saps. The applicability of ^{32}P-labeled dsRNA for diagnosing specific sequences of plant virus RNAs is discussed.

In a recent review, Kaper and Waterworth (15) gave a list of 59 cucumber mosaic virus (CMV) isolates; these were considered CMV strains because some biological and chemical differences do occur among them. Serology has been useful for routine indexing of plants for CMV infection. However, antigenic relatedness ranging from nonidentity to near identity between isolates of CMV and certain isolates of the other two members of the cucumovirus group (tomato aspermy virus [TAV] and peanut stunt virus [PSV]) limits the use of serology for classifying certain strains as members of this group (15).

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CMV particles contain at least four RNA segments designated RNA 1–4 in order of decreasing molecular weight. The largest three are the minimum required for infectivity (4). In certain cases, CMV particles also contain a second replicating entity designated the CMV-associated RNA (CARNA-5). Interaction between the viral genome, the virus-dependent satellitelike CARNA-5, and the host influences virus multiplication, symptoms, and infectivity (8,15,29,32). The CARNA-5 from different CMV strains were shown to lack sequence homology with their genomic RNAs (3,15,17,20). The inadequacies of serology for virus typing and the association of unique RNA sequences with pathogenicity indicated the need of nucleic acid hybridization techniques for cucumovirus diagnosis. Hybridization techniques that employ complementary DNA (cDNA) probes have been applied successfully for specific diagnosis of genomic viral sequences (5,7,16,17,30), satellite RNAs

(6,24), and viroids (25,27).

This paper reports on the use of a ^{32}P-labeled double-stranded (ds) CARNA-5 probe isolated from *Pachystachys coccinea* (1) for the detection of CARNA-5-like sequences in plant extracts. Using this probe for Northern blot hybridization, two sizes of molecules resembling dsCARNA-5 were detected in CMV-infected plants of *Nicotiana glauca*.

MATERIALS AND METHODS

Plants, virus strains, and serology. Plants of *N. glauca* naturally infected with cucumoviruses were collected in two local habitats. Standard dsCARNA-5 was purified from plants of *P. coccinea* infected with CMV. The presence of cucumoviruses in leaf homogenates prepared with an extraction buffer described by Owens and Diener (26) was tested by the double antibody sandwich form of ELISA by using an antiserum to Price No. 6, CMV strain (18).

Isolation of dsRNA. Plant samples were homogenized in 4% (w/v) paraaminosalicylic acid (PASA), phenol extracted, and dsRNA was purified on a CF-11 column essentially as described by Bar-Joseph et al (1) and Morris and Dodds (23).

Gel electrophoresis. Nucleic acids were fractionated for 16 hr at 45 mA on a vertical 5% polyacrylamide gel (19) or a horizontal 1% agarose slab gel in E buffer (40 mM tris-HCl, pH 7.8, 20 mM Na-acetate and 1 mM EDTA) for 3 hr at 100 mA.

Purification of dsCARNA-5. Following electrophoretic fractionation of dsRNA in polyacrylamide gel, the area of the gel containing the dsCARNA-5 (as visualized by ethidium bromide staining) was excised and placed in a dialysis bag containing a minimal volume of E buffer. Elution of RNA from the gel was carried out by applying a voltage of 80 V for about 15 min. The eluted RNA was ethanol-precipitated and redissolved in 50 μl of water.

Polynucleotide kinase-mediated ^{32}P-labeling. Labeling of the purified dsCARNA-5 fraction with [^{32}P] was carried out mainly as described by Maizels (21). A pellet of ~1 μg dsRNA was resuspended in 5 μl of hydrolysis buffer (50 mM tris-HCl, pH 9.5) and introduced into a siliconized capillary tube (10 μl) that was sealed at each end. The capillary was heated in a water bath at 90 C for 20 min and its content was transferred to a plastic tube (1.5 ml) to which 2 μl of reaction buffer (10×), 2.5 μl [^{32}P]-γ-ATP (25 μCi, 3,000 Ci/mM, Amersham, Buckinghamshire, England) and 1 μl (four units) of polynucleotide kinase (Boehringer, Mannheim, West Germany) were added in a final volume of 20 μl. The mixture was incubated for 2 hr at 37 C and the reaction was terminated by adding 1.5 μl of 4 M sodium acetate. Precipitation of RNA after adding 5 μl of *Escherichia coli* tRNA (6 mg/ml) and 10 μl ethanol was carried out at −20 C for 16 hr. After centrifugation, the pellet was washed several times by dissolving it in 50 μl of H_2O followed by an ethanol precipitation to remove free nucleotides. The labeled RNA was finally dissolved in 50 μl of H_2O. Specific activity of about 2×10^6 cpm per 1 μg of dsRNA was obtained.

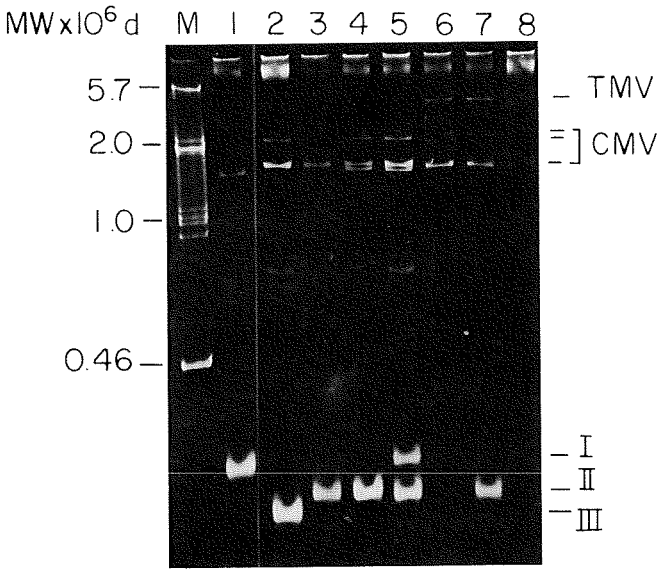

Fig. 1. Double-stranded (ds)RNA patterns of naturally infected plants of *Nicotiana glauca*. The dsRNAs from plants of *N. glauca* infected with cucumoviruses (CMV) (lanes 1–8) or a mixed infection of cucumoviruses and tobacco mosaic virus (TMV) (lanes 6–8) were purified (1) and fractionated by electrophoresis in 5% polyacrylamide gel and stained by ethidium bromide. Molecular-weight size markers of dsRNA (2) were run in parallel (M).

Northern blots of dsRNA. The transfer of dsRNA patterns from agarose gel to nitrocellulose paper was performed as in (31) except that the gel was briefly soaked in alkaline solution (0.1 M NaOH) and then neutralized in 1 M tris-HCl (pH 7.4) immediately before transfer (1).

Hybridization. The [^{32}P] dsCARNA-5 probe (about 2×10^5 cpm) was heated to 100 C for 2 min followed by rapid cooling on ice before adding it to the hybridization reaction. Hybridization was carried out at 42 C in a buffer containing 50% formamide, 4×SSC, 1×Denhardt, and 50 mM Na phosphate buffer (pH 6.5) essentially as described by Thomas (31).

RESULTS

Isolation and analysis of dsRNA in *N. glauca*. Double-stranded (ds)RNA was isolated from several *N. glauca* plants in which CMV was detected with the ELISA test (18). The dsRNA was fractionated by electrophoresis in 5% polyacrylamide gel (Fig. 1). The patterns obtained revealed infection with cucumoviruses (Fig. 1, lanes 1–5) and mixed infections of cucumoviruses and tobacco mosaic virus (TMV) (Fig. 1, lanes 6–8). In addition to the genomic dsRNA of cucumoviruses already described (10,15), three types of low-molecular-weight dsRNA species with the following molecular weights were observed: (type I) 0.27×10^6 daltons, (type II) 0.22×10^6 daltons, and (type III) 0.19×10^6 daltons (Fig. 1, lanes 1, 3, and 2, respectively). Type II had an estimated molecular weight similar to *P. coccinea* dsCARNA-5 (1) and was found in 26 of 31 of the dsCARNA-5-like isolates. Four other isolates belonged to type I, and one to type III. The presence of both types I and II is shown in Fig. 1, lane 5, and cucumovirus infections not associated with low-molecular-weight RNA are shown in Fig. 1, lanes 6 and 8.

Detection of dsCARNA-5 by blot hybridization. In order to ascertain which of these low-molecular-weight dsRNA species is related to CARNA-5, a ^{32}P-labeled probe made of purified dsCARNA-5 from *P. coccinea* was prepared. Samples of dsRNA from *N. glauca* containing the various types of low-molecular-weight bands were fractionated by electrophoresis in 1% agarose gel and visualized by ethidium bromide staining (Fig. 2A). It should be noted that the differences in mobilities of the three types of low-molecular-weight dsRNAs in the agarose gel were very small and much more difficult to distinguish compared to the high resolution of the polyacrylamide gel (Fig. 1). The dsRNA patterns were blot-transferred to a nitrocellulose sheet and subjected to hybridization with the labeled dsCARNA-5 probe (Fig. 2B). Except for the larger dsRNA species of molecular weight 0.27×10^6 (Fig. 2B, lane 6), the two other types of molecular weights 0.22 (Fig. 2B, lanes 1, 3, and 4) and 0.19×10^6 (Fig. 2B, lane 7) hybridized positively with the dsCARNA-5 probe.

These results were also corroborated by hybridizing the dsCARNA-5 probe with samples of dsRNA spotted onto a nitrocellulose sheet (Fig. 3A). The samples that hybridized positively with the probe contained either the 0.22×10^6-dalton (type II) species (Fig. 3A, spots 2, 7, and 8) or both types II and I (0.27×10^6 daltons) (Fig. 3A, spots 5 and 6). Samples containing type I alone did not hybridize with the dsCARNA-5 probe (Fig. 3A, spots 3 and 4). Hence, it seems that this larger type of satellite RNA is of a different origin.

Using the ^{32}P-labeled dsCARNA-5 probe, dsRNA could be detected in as low as 1:256 dilution of the original concentration of a positive sample (Fig. 3B), while in a gel stained with ethidium

bromide the limit of detection of the same set of dilutions was only 1:16 (*unpublished*). Thus, this hybridization technique proved to be more sensitive than gel electrophoresis.

Detection of CARNA-5 in plant sap by spot hybridization. We have further tested the possibility of using the spot hybridization technique for detection of CARNA-5 sequences directly in plant sap. Crude saps were prepared from *N. glauca* plants infected with cucumoviruses, spotted onto a nitrocellulose sheet, and hybridized with a radio-labeled dsCARNA-5 probe (Fig. 3C). Saps containing the type I RNA species failed to hybridize with the dsCARNA-5 probe (Fig. 3C, spots 1 and 2). Saps containing CMV without CARNA-5 (Fig. 3C, spots 5 and 6) reacted similarly negatively. Samples containing either type II (Fig. 3C, spots 4 and 9), or type III (spot 7) positively hybridized with the probe. In this way, we were able to detect specific CARNA-5 sequences in crude saps of plants.

The dot spot hybridization technique probing with ^{32}P-labeled dsCARNA-5 was applied for testing the presence of CARNA-5 in plant extracts. *N. glutinosa* and cucumber leaves were mechanically inoculated with several CMV isolates from *N. glauca*. A sample of each plant was tested for the presence of CARNA-5 by the dot spot hybridization technique and by gel electrophoresis of dsRNA extracts. In six of 28 *N. glutinosa* samples, positive hybridization with the *P. coccinea* ^{32}P-labeled dsCARNA-5 probe was observed. These samples positively reacted with CMV antiserum in ELISA, and dsCARNA-5 was also visualized in stained polyacrylamide gels. Four samples of *N. glutinosa* that contained the slower migrating band, all the cucumber plants, and the healthy controls of *N. glutinosa* failed to hybridize with the dsCARNA-5 probe. We were thus able to monitor plants containing CARNA-5.

DISCUSSION

The present work is intended primarily to show the applicability of hybridization methods in which polynucleotide kinase-mediated ^{32}P-labeled dsRNA probes are used for the rapid and simple screening of plant virus RNA species. The main advantages of using dsRNA as a probe for hybridization are its relatively simple isolation procedure from many virus infected plants (1,23), its distinct banding in polyacrylamide gel, and its rapid elution from the gel. Moreover, since dsRNA is not sensitive to residual RNAse activities, the extraction of intact uncontaminated genomic or subgenomic populations can be readily carried out. In addition, a dsRNA probe will hybridize to genomic as well as replicative forms of viral RNA, thereby increasing the sensitivity of the detection procedures. It should be noted that dsCARNA-5 is found in relatively high amounts in the plant tissue and, therefore, is easily isolated and used as a probe. The applicability of this technique in other viral systems will depend on the availability of ~1-μg quantities of purified dsRNA. Most of the cucumovirus isolates collected from *N. glauca* grown in the Beer Sheva area reacted serologically with a CMV antiserum. The isolates contained a satellite RNA with migration patterns similar to that of *P. coccinea* dsCARNA-5 and were found by Northern blot hybridization to bind ^{32}P-labeled *P. coccinea* dsCARNA-5. On the other hand, three of the cucumovirus isolates from *N. glauca* collected at Miqveh Yisrael and one isolate from Beer Sheva contained the slower-migrating dsRNA species that did not hybridize with CARNA-5 from *P. coccinea*. Previous analysis of CARNA-5 originating from the U.S., France, Japan, and Australia showed only small differences in nucleotide sequences (9,12,28, and R. H. Symons as cited in 15). The dsRNA probe effectively hybridized with both the dsRNA extracts and with plant sap diluted with an extraction buffer (26). The same buffer was also found useful for the serological testing of cucumoviruses in ELISA. This enabled

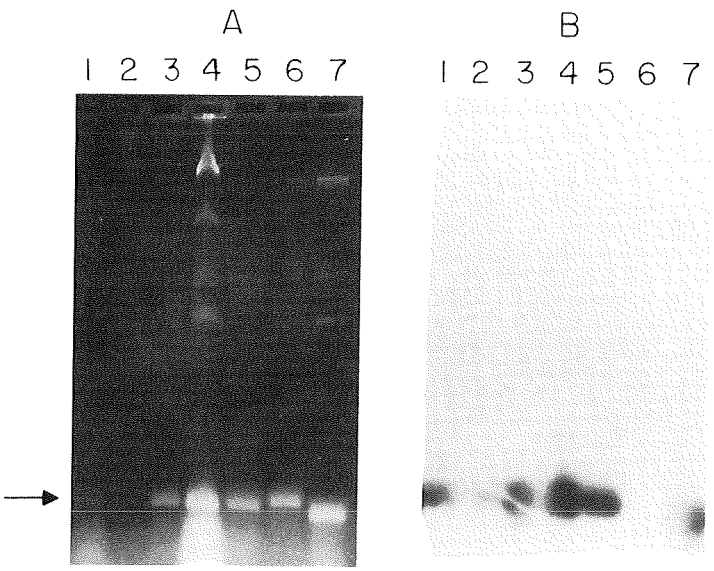

Fig. 2. Hybridization of dsRNA blots with a labeled dsCARNA-5 probe. The dsRNAs from *Nicotiana glauca* plants containing low-molecular-weight species types I (lane 6), II (lanes 1, 3, and 4), and III (lane 7) were fractionated in a 1% agarose gel. A dsRNA sample containing both types I and II was run in lane 5 and a purified dsCARNA-5 in lane 2. The gel was stained with ethidium bromide (A) and blotted onto nitrocellulose paper, which was subjected for hybridization with a ^{32}P-labeled-dsCARNA-5 probe (B).

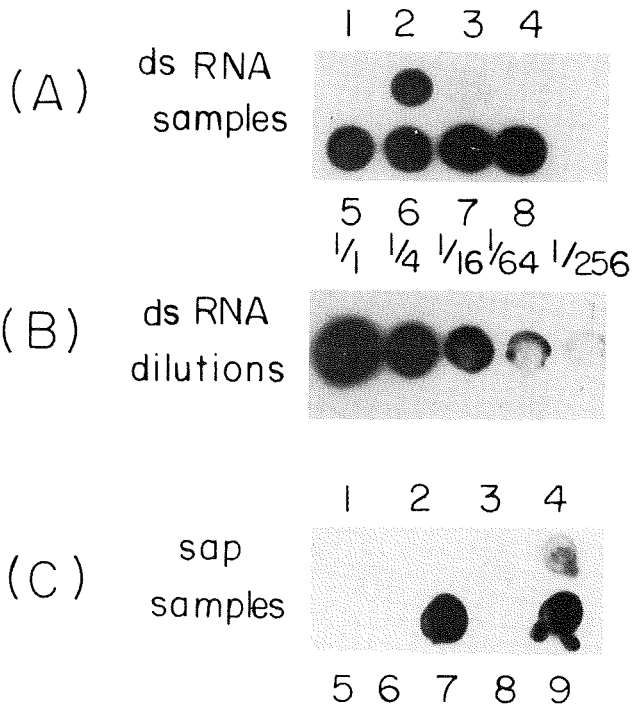

Fig. 3. Detection of CARNA-5 sequences by spot hybridization with a labeled dsCARNA-5 probe. A radio-labeled dsCARNA-5 was used as a probe for hybridization with the following samples spotted onto nitrocellulose paper. **A,** dsRNA samples from plants of *Nicotiana glauca* containing low-molecular-weight type I (spots 3 and 4), type II (spots 2, 7, and 8), and a mixture of types I and II (spots 5 and 6) were spotted onto nitrocellulose: Spot 1 is of dsRNA from a healthy plant. **B,** A sample of dsRNA, which positively hybridized with a dsCARNA-5-probe, was serially diluted in water (as indicated in the figure) and a sample of each dilution was spotted onto the nitrocellulose sheet. **C,** Saps from plants infected with cucumoviruses containing various types of low-molecular-weight dsRNA were prepared according to Owens and Diener (26) and 3-μl samples of each were spotted onto nitrocellulose following short denaturation-neutralization treatments (1). Sap samples, containing low-molecular-weight type I (spots 1 and 2), type II (spot 4), and type III (spot 7), CMV-infected plants not containing any low-molecular-weight dsRNA (spots 5 and 6), a healthy plant (spots 3 and 8) and *Pachystachys coccinea* infected with CMV containing CARNA-5 (spot 9).

viral diagnosis by combining ELISA, and the identification of CARNA-5 by spot hybridization in plant sap. The sap spot hybridization experiment indicated that ^{32}P-labeled dsCARNA-5 from *P. coccinea* did bind to sap components of six plants of *N. glutinosa* that contained type II (0.22×10^6) ds satellite RNA. Hybridization did not occur with sap spots obtained from cucumber plants inoculated with the same isolates. These plants did not contain a dsCARNA-5 after dsRNA extraction and gel electrophoresis. This confirms previous experiments (11), which indicated that the host plant has a role in determining satellite association with CMV infection. Sap from plants of *N. glutinosa* that contained the type I low-molecular-weight dsRNA (0.27×10^6 daltons) reacted positively with CMV antiserum in the ELISA test, but this satellite did not hybridize with labeled dsCARNA-5 from *P. coccinea*, thus indicating the lack of sequence homology between these two satellites. Host range studies of one type I isolate indicated that it differs from PSV (22). A satellite RNA slower migrating than CARNA-5 has been found recently in PSV infected plants (13). The antigenic and biological properties of the cucumoviruses overlap, so as to make typing and identification of many strains difficult (15). Further characterization of the *N. glauca* isolates will be needed in order to precisely classify them among the cucumoviruses.

The suggestions of involvement of CARNA-5 in CMV pathogenicity (14,29,32) and the involvement of dsCARNA-5 in plant defense (8) warrants further studies on both the quantitative aspects of CARNA-5 accumulation and the qualitative aspects of genomic composition of other low-molecular-weight RNAs associated with infections of plants by cucumoviruses. The hybridization method described in this work is expected to facilitate future studies on these topics.

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