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Sensitive Detection of Cucumber Mosaic Cucumovirus RNA with Digoxigenin–Labeled cRNA probe

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To nonradioactively detect cucumber mosaic cucumovirus (CMV) RNA with high sensitivity, we tested the use of CMV RNA–specific digoxigenin (DIG)–labeled ribonucleic acid probe, which was *in vitro* transcribed, in dot–blot, northern blot and microplate hybridization. The probe could detect up to 10 fg of CMV RNA by dot–blot hybridization with a chemiluminescent detection. Northern blot analysis showed that the riboprobe hybridizes to all CMV genomic RNAs in the total RNAs extracted from the inoculated leaves of Japanese radish without nonspecific reactions against mock–inoculated samples. Furthermore, accumulation of CMV RNA in the inoculated leaves of bottle gourd could be monitored with dot–blot and microplate hybridization. Thus, we demonstrated that the hybridization methods with nonradioactive riboprobe are applicable to highly sensitive and specific detection of CMV RNA not only in purified virion but also in total RNA extracted from host plants infected with CMV.

INTRODUCTION

Cucumber mosaic cucumovirus (CMV) is well–known as one of the most prevalent plant virus which gives serious damage to various host plants. CMV is a tripartite icosahedral virus and is the type member of the *Cucumovirus* in the family of *Bromoviridae* (reviewed in Palukaitis *et al.*, 1992). Its genome is divided into three single–stranded positive sense RNAs designated RNA1, RNA2 and RNA3 in decreasing order of molecular weight. We are now studying the molecular basis of the host–specificity of CMV. To analyze the kinetics of accumulation of CMV RNAs in host plants, we developed nonradioactive assay methods using a CMV–specific *in vitro* transcribed ribonucleic acid probe that was labeled with digoxigenin (DIG). In this paper, we describe qualitative detection of all genomic RNAs of CMV in the total RNA purified from the inoculated cotyledons of Japanese radish (*Raphanus sativus* L.) with northern blot hybridization, and quantitative detection of CMV RNAs in the total RNA from those of bottle gourd (*Lagenaria siceraria* Standl.) with dot blot and microplate hybridization.

MATERIALS AND METHODS

Viruses and Plants

CMV–KM (Takeshita and Takanami, 1997), CMV–Y (Tomaru and Hidaka, 1960) and CMV–D8 (Takeshita and Takanami, 1997) were used in this study. Virus was propagated in *Nicotiana tabacum* L. cv. Xanthi–nc and purified essentially according to Takanami

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(1981). Japanese radish plants were maintained in an air-conditioned green house at 22 -28 °C and bottle gourd plants were in an environmentally controlled growth chamber at 25 °C with a 14-hr light/10-hr dark cycle. Fully expanded cotyledons of the plants were inoculated with virions purified from the tobacco plants infected with CMV.

DIG-labeled riboprobe

A nonradioactive RNA probe was *in vitro* transcribed from a clone pCD3XN (3 μg), which contains the *Xho*I-*Not*I fragment in the 3' terminal region of a full length cDNA clone of CMV-D8 RNA3 (Takeshita *et al.*, 1998), using digoxigenin-11-uridine-5'-triphosphate (DIG-11-UTP, Boehringer Mannheim). After digestion with ribonuclease free deoxyribonuclease I (Takara) and ethanol precipitation of the template DNA, the RNA probe was suspended in 50 μl of H_2O. The amount of the RNA probe produced was evaluated by 1.0% agarose gel electrophoresis with ethidium bromide staining. The probe is complementary to the 379 nucleotides of the 3' region of CMV-D8 and is designed to specifically hybridize to the conserved 3' sequences common to all the three CMV RNA genomes.

Hybridization

Details of the procedures for dot blot and northern hybridization and detection were described previously (Takeshita *et al.*, 1998). Microplate hybridization was carried out essentially according to Inouye and Hondo (1990). Total RNAs purified from the inoculated leaves of bottle gourd plants were diluted in microplate wells (Nunc, Roskilde, Denmark) in 200 μl of 10 mM sodium phosphate, pH7.0, containing 10 mM EDTA and 1.5 M NaCl. After being sealed with adhesive tape, the plate was incubated at 37 °C for 2 hr. The wells were washed three times with 10 mM sodium phosphate buffer, pH7.2, 0.14 M NaCl, 0.1% Tween 20 (PBST). Then, 100 μl of solution containing the heat-denatured DIG-labeled riboprobe (0.25 μl/ml), 50% formamide, 0.75 M NaCl, 5 mM sodium phosphate, pH7.0, 1 mg/ml of total yeast RNA, 0.1% Tween 20, and 5 mM EDTA was added. The plate was incubated at 65 °C overnight. After rinsing the plate with PBST, 100 μl of an antibody against DIG that was conjugated with alkaline phosphatase (diluted 1:1000 in PBST, Boehringer Mannheim) was added to each well. The plate was incubated at 37 °C for 2 hr and rinsed as described above. Then, disodium-*p*-nitrophenyl phosphate (1 mg/ml) suspended in 250 μl of diethanolamine buffer, pH. 9.8, was added to each well. The plate was further incubated at 30 °C for 2 hr. Absorbance at 405 nm of each well was measured with MTP-120 MICROPLATE READER (CORONA ELECTRIC).

RESULTS AND DISCUSSION

The results of the dot-blot hybridization demonstrated that the riboprobe could detect up to 10 fg of CMV-D8 RNA (Fig. 1a). In this assay, chemiluminescent detection using CSPD® was at least 100 fold more sensitive than colorimetric detection using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Figs. 1a, 1b). Furthermore, extention of exposure time to X-ray film did not significantly increase the detection limit of the target RNA with the chemiluminescent detection.

Northern blot analysis revealed that the riboprobe specifically hybridized to CMV

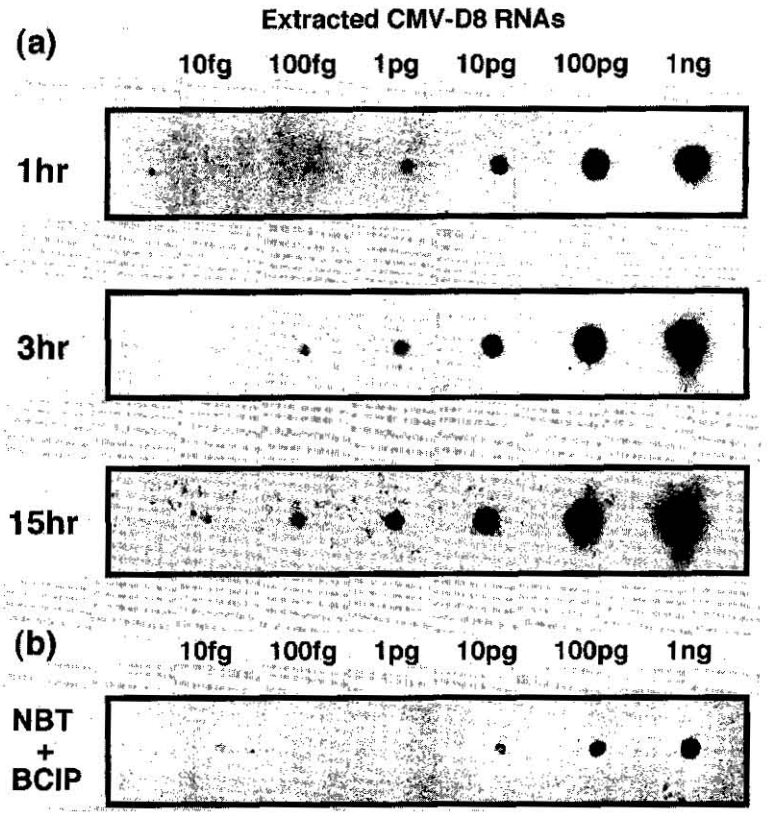

Fig. 1. Sensitivity of the CMV-specific digoxigenin (DIG)-labeled RNA probe. Various amounts of CMV-D8 RNA were dot blotted on to nylon membranes. CMV RNAs were hybridized with the riboprobe and detected using (a) CSPD® as a chemiluminescent reagent and (b) NBT and BCIP as colorimetric reagents.

genomic RNAs in the total RNAs extracted from the inoculated leaves of Japanese radish (Fig. 2). In addition, the riboprobe did not gave any signals against the total RNAs obtained from the mock-inoculated leaves.

To know the kinetics of CMV RNA accumulation in host plant, total RNAs were purified from the inoculated leaves of bottle gourd and subjected to dot blot and microplate hybridization at different days post inoculation (p.i.). As shown in Figs. 3a, 3b, CMV RNAs were detectable at 2 days p.i. irrespective of CMV isolates. The accumulation of CMV-Y RNAs was lower level than that of CMV-KM and CMV-D8 RNAs and reached a plateau at 3 days p. i. This finding was also consistent with the results obtained from microplate hybridization that can specifically detect CMV RNAs in total RNAs extracted from the inoculated leaves (Fig. 4). CMV-KM and CMV-D8 induce enlarged chlorotic spots on the inoculated cotyledons, whereas CMV-Y elicites pin-pointed necrotic lesions

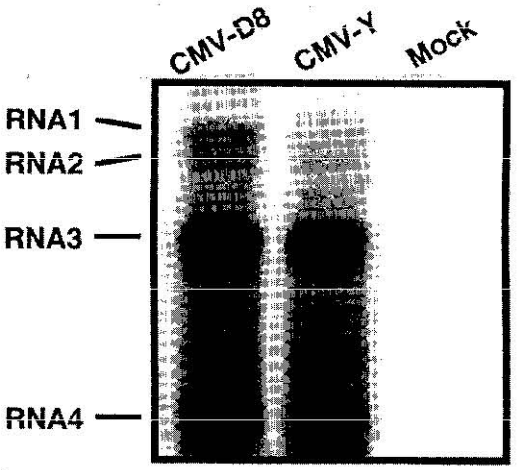

Fig. 2. Northern blot analysis of CMV RNAs in the cotyledons of radish plants inoculated with CMV–D8 and CMV–Y at a concentration of 1 mg/ml. The leaves were detached for analysis one week p.i. Total RNAs were extracted from all of the cotyledons and separated by agarose gel electrophoresis, then CMV RNAs were detected with the CMV–specific DIG-labeled RNA.

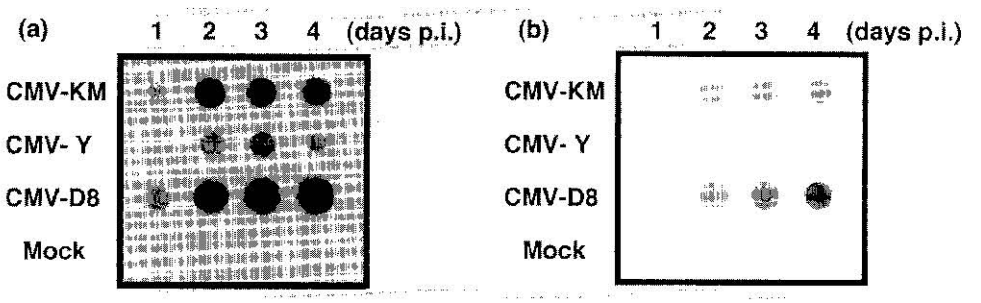

Fig. 3. Accumulation of CMV RNAs in the cotyledons of bottle gourd plants inoculated with CMV–KM, CMV–Y or CMV–D8 at a concentration of 0.2 mg/ml. The cotyledons were detached 1, 2, 3 and 4 days p.i. CMV RNAs extracted from the cotyledons were dot blotted and hybridized with the CMV–specific DIG–labeled RNA. The hybridization signals were visualized using (a) CSPD® as a chemiluminescent reagent and (b) NBT and BCIP as colorimetric reagents.

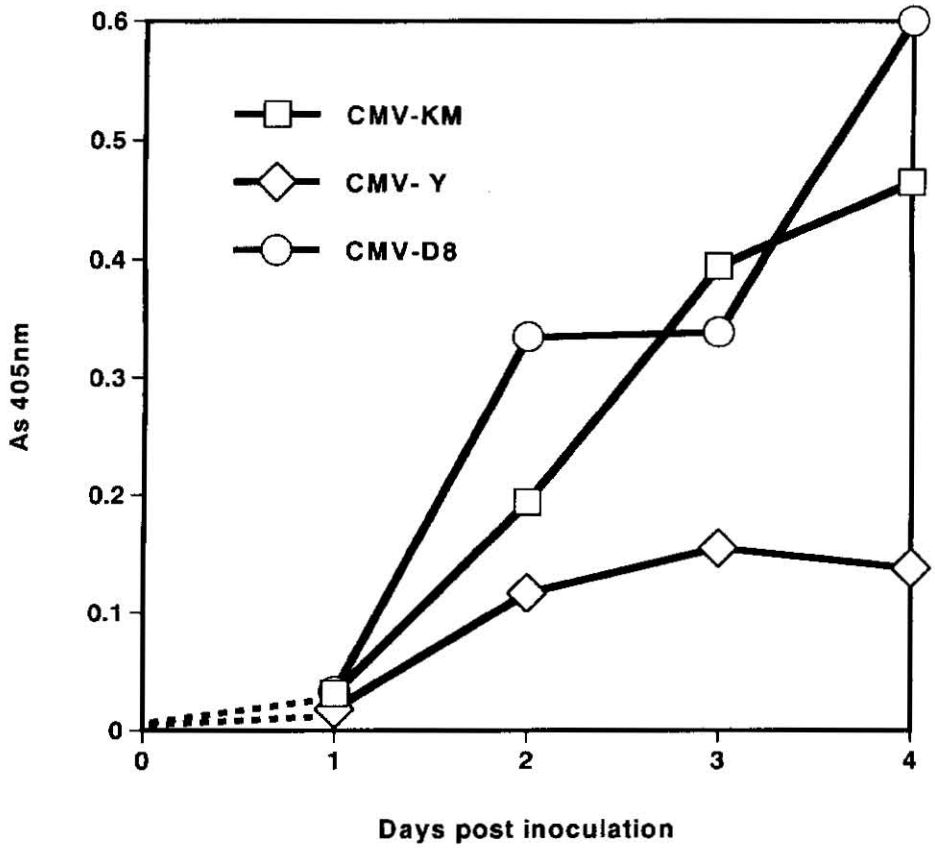

Fig. 4. Analysis of CMV RNA accumulation in the cotyledons of bottle gourd with microplate hybridization. The cotyledons of the plants were inoculated with CMV–KM, –D8 or –Y at a concentration of 0.2 mg/ml. RNA samples taken 1–4 days p.i. were subjected to microplate hybridization as described in the Text.

(will be described elsewhere). The results obtained from the dot blot and microplate hybridization reflected the differences in symptom phenotype in the inoculated leaves.

There have been several reports on the use of DIG–labeled cDNA or *in vitro* synthetic cRNA probes for detection of viral RNAs (Mas *et al.*, 1993; Dietzgen *et al.*, 1994; Saito *et al.*, 1997; Takanami *et al.*, 1999; Uchiba *et al.*, 1999). Recently, Takanami *et al.*, (1999) reported the specific detection of CMV subgroups using DIG–labeled synthetic oligo–deoxyribonucleotide probes. The probes could detect up to 10 pg of CMV RNA without cross–reactions between the subgroups I and II. In this study, dot–blot, northern blot and microplate hybridization using the DIG–labeled synthetic cRNA probe could also detect CMV RNA with high specificity and sensitivity. The nonradioactive assay methods would greatly facilitate the study on the analysis of CMV RNA accumulation and

translocation in host plants.

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