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Uchiba, Takayuki

Laboratory of Plant Pathology, Graduate School of Bioresource and Bioenvironmental Sciences,
Kyushu University

Takeshita, Minoru

Laboratory of Plant Pathology, Graduate School of Bioresource and Bioenvironmental Sciences,
Kyushu University

Takanami, Yoichi

Laboratory of Plant Pathology, Graduate School of Bioresource and Bioenvironmental Sciences,
Kyushu University

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Quantitative Detection of Cucumber Mosaic Cucumovirus RNA with Microplate Hybridization Using Digoxigenin-Labeled Oligo-Deoxyribonucleotide Probe

Takayuki Uchiba, Minoru Takeshita, and Yoichi Takanami

Laboratory of Plant Pathology, Graduate School of Bioresource and
Bioenvironmental Sciences, Kyushu University, Hakozaki,
Higashi-ku, Fukuoka 812-8581, Japan

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A simple microplate hybridization method was developed for subgroup-specific and quantitative detection of RNA of cucumber mosaic cucumovirus (CMV). Digoxigenin (DIG)-labeled, synthetic oligo-deoxyribonucleotides were used as the probes for hybridization. CMV RNAs were adsorbed to microplate wells and then hybridized with the probes specific for the respective subgroups of CMV. Under optimal conditions, about one ng of CMV RNA was detected without any nonspecific reactions. When total RNAs extracted from tobacco plants infected with CMV-Y, CMV-m2 or mock-inoculated were subjected to the hybridization, we found no cross-reactions between viral RNAs of subgroup I and II and no nonspecific reactions against healthy controls. The microplate hybridization method had several advantages over conventional dot blot hybridization methods.

INTRODUCTION

Cucumber mosaic cucumovirus (CMV) is a small spherical plant virus and is the type member of *Cucumovirus* in the family of *Bromoviridae*. CMV has a tripartite RNA genome consisting of RNA1, RNA2, and RNA3 (Peden and Symons, 1973); a subgenomic coat protein messenger RNA (RNA4) is generated during replication of RNA3 (Schwinghamer and Symons, 1974). CMV is divided into subgroup I and II based on serological property and differences in nucleotide sequences (Palukaitis *et al.*, 1992). We are now investigating the molecular basis of a phenomenon in plant virus infection, known as cross-protection, using various CMV strains. In the course of this study, we need to develop methods that could discriminate subgroup I and II of CMV RNAs in doubly infected cells or leaf tissues. We have already developed a method to specifically detect CMV strains belonging to the respective subgroups with dot blot and northern blot hybridization using digoxigenin(DIG)-labeled synthetic oligo-deoxyribonucleotide probes (Takanami *et al.*, 1999).

Inouye and Hondo (1990) developed a new hybridization method for detection of varicella-zoster virus by directly adsorbing PCR-amplified viral DNA fragments onto polystyrene microplate wells. Thereafter, a number of reports describing the "microplate hybridization" method have accumulated and availability of the method has been confirmed (Ruiz *et al.*, 1995). Almost all of these procedures contained the use of PCR-amplified cDNA as targets. In this study, a microplate hybridization technique was developed for subgroup-specific detection of CMV RNAs using DIG-labeled, synthetic oligo-deoxyribonucleotide probes complementary to the specific regions in the viral

genome RNAs of the respective subgroups. The immobilized RNA was hybridized with the probes, then the hybridization signal was detected by alkaline phosphatase-conjugated antibody, as in an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Viruses and genomic RNA extraction

CMV-Y (Tomaru and Hidaka, 1960) belonging to subgroup I and CMV-m2 (Takanami *et al.*, 1998) belonging to subgroup II were maintained in *Nicotiana tabacum* L. cv. Xanthi-nc in a greenhouse. Virus was purified as described before (Takanami, 1981). Viral RNA was extracted as follows. Purified virus was incubated at 50°C for 5 min in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), then RNA was extracted with an equal volume of phenol:chloroform (1 : 1) mixture, and precipitated with 2.5 volumes of ethanol. Total RNA in the infected tobacco leaves were extracted according to Takeshita *et al.* (1998).

DIG-labeled probes

DIG-labeled oligo-deoxyribonucleotide probes Y-2 and m2-2 were complementary to the 3' non-translated regions of CMV-Y (RNA3 : 2132-2175 nt) and CMV-m2 (RNA3: 2113-2157 nt), respectively. The details of the probes will be described elsewhere (Takanami *et al.*, 1999).

Microplate hybridization

Hybridization was carried out as follows. The purified CMV RNA was diluted in 0.2 ml of 10 mM sodium phosphate, pH 7.0, and 10 mM EDTA, containing various concentrations of NaCl and added to the each well of microtiter plate for immunosorbent assay (Nunc-immuno plate). After incubated at 37°C for 2 hr, the wells were rinsed once with phosphate-buffered saline containing 0.1% Tween 20 (PBST). Then 0.1 ml solutions containing the probes (20 ng/ml), 50% formamide, 0.75 M NaCl, 5 mM sodium phosphate, pH 7.0, 0.1 mg of total yeast RNA, 0.1% Tween 20, and 5 mM EDTA were added. The microplate was incubated in a hybridization oven at various temperatures as mentioned below for more than 16 hr. After the wells were rinsed three times with PBST, 0.1 ml of alkaline-phosphatase-conjugated anti-DIG- γ -globulin, Fab fragment (Boehringer Mannheim), that was diluted to 1 : 5000 in PBST was added. After incubated at 37°C for 3 hr, the wells were rinsed with PBST three times. For each well, 0.1 ml of 1 mg/ml *p*-nitrophenyl-azo-phosphate diluted in diethanol amine, pH 9.8, was added. The plate was sealed, and then incubated at 37°C for 2 hr. Absorbance at 405 nm of each well was determined with a microplate reader every thirty minutes after incubation.

RESULTS

Optimum NaCl concentration for adsorption of CMV RNA to microplate wells

To determine the optimum concentration of NaCl for immobilizing CMV RNA to the well, fixation step was carried out at various NaCl concentrations. In this experiment, each well contained 10 ng of CMV-Y or CMV-m2 RNA, and the RNA was hybridized with

probe Y-2 or m2-2. As shown in Table 1, fixation at 2.5 M NaCl gave the highest hybridization signals. Potassium chloride or ammonium sulfate could also be used to fix CMV RNA (data not shown).

Table 1. Effect of various NaCl concentrations on immobilization of CMV RNA to microplate.

| NaCl concentrations | Probe / Sample RNA | |
|---------------------|--------------------|---------------|
| | Y-2 / CMV-Y | m2-2 / CMV-m2 |
| 1.0M | 0.021 | 0.027 |
| 1.5M | 0.172 | 0.103 |
| 2.0M | 0.241 | 0.126 |
| 2.5M | 0.261 | 0.190 |
| 3.0M | 0.144 | 0.160 |

Absorbance at 405 nm was measured after colorimetric reaction for 2 hr. Each well contains 10 ng of CMV RNA.

Hybridization temperature

To determine the optimum temperature for the hybridization reaction between target RNAs and the DIG probes, hybridization was carried out at different temperatures. In this experiment, each well contained 100 ng of CMV-Y or CMV-m2 RNA. Both probes Y-2 and m2-2 gave the highest values of absorbance when hybridization was carried out at 45 °C (Table 2). The sensitivity of probe m2-2 was lower at higher or lower temperature than at 45 °C compared with that of probe Y-2.

Table 2. Optimum hybridization temperature for detection of CMV RNA.

| Hybridization temperatures | Probe / Sample RNA | |
|----------------------------|--------------------|---------------|
| | Y-2 / CMV-Y | m2-2 / CMV-m2 |
| 43 °C | 0.846 | 0.696 |
| 45 °C | 1.663 | 1.634 |
| 47 °C | 1.649 | 0.720 |

Absorbance at 405 nm was measured after colorimetric reaction for 2 hr. Each well contains 100 ng of CMV RNA.

Sensitivity of microplate hybridization

To know the detection limit of CMV RNA with microplate hybridization, CMV RNA was serially twofold diluted and adsorbed to the microplate. Adsorption of CMV RNA and hybridization reaction were performed at the optimum conditions described above. About 1 ng of CMV RNA could be detected, and probes Y-2 and m2-2 showed almost equal sensitivity to the RNAs of the respective CMV strains (Fig. 1).

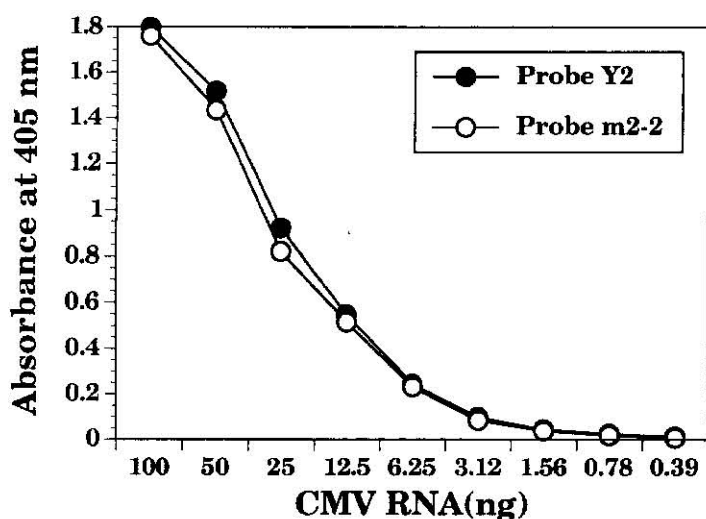


Fig. 1. Sensitivity of the microplate hybridization method for detection of CMV RNA. Purified genomic RNA of CMV-Y or CMV-m2 was serially diluted in 2-fold steps from 100 ng to about 0.39 ng/0.2 ml. RNA was adsorbed to microplate wells, and hybridization was carried out using the DIG-labeled probe. Absorbance values were measured after colorimetric reaction for 2 hr.

Specificity of microplate hybridization

RNAs of CMV-Y and CMV-m2 were immobilized in wells of microplate (100 ng/well) and the hybridization reactions were carried out using the both probes.

As shown in Table 3, the both probes hybridized only with the RNA of the respective CMV strains. Furthermore, when total RNAs extracted from tobacco plants infected with CMV-Y, CMV-m2 or mock-inoculation were subjected to the hybridization, we found no cross-reactions between viral RNAs of subgroup I and II and no nonspecific reactions against healthy controls.

Table 3. Subgroup specific detection of CMV RNA.

| Probes | Purified CMV RNA | | Total RNA from tobacco leaves | | |
|--------|------------------|-------|-------------------------------|-----------------|------------------|
| | Y | m2 | H ^{a)} | Y ^{b)} | m2 ^{b)} |
| Y-2 | 1.673 | 0.004 | 0.001 | 0.445 | 0.000 |
| m2-2 | 0.005 | 1.644 | 0.000 | 0.000 | 0.525 |

Absorbance at 405 nm was measured after colorimetric reaction for 2 hr. Purified CMV RNA (100 ng each) or total RNAs (extracted from about 1.25 mg of infected tobacco leaf) were adsorbed. a): Total RNA were extracted from healthy tobacco leaf. b): Total RNA were extracted from tobacco leaves infected with CMV-Y or CMV-m2.

DISCUSSION

The microplate hybridization method described here has several advantages over conventional dot blot methods. First, the microplate can be handled much more easily than the dot blot membrane. For instance, washing of the microplate solid phase is more rapid, as it is in the ELISA. Furthermore, blocking procedures that are indispensable for dot blot hybridization on membranes were not required. Second, a specific RNA can be detected not only qualitatively but also quantitatively by reading the optical absorbance values using an automated microplate reader.

Generally, hybridization reaction between probes and target nucleic acid is considered to be enhanced at lower hybridization temperature though nonspecific hybridization would increase. Our data concerning the optimal hybridization temperature, however, were not the case. We speculate that the target RNA adsorbed to microplate at only one end (3' or 5') of the strand might form secondary and/or tertiary structures more easily at lower temperature so that hybridization between RNA and probes would be inhibited. It is well known that the 3'-noncoding regions of CMV RNA where the target sequences of the probes were present can form tRNA-like structure (Wilson *et al.*, 1981; Joshi *et al.*, 1983).

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