Specific Detection of Subgroups I and II of Cucumber Mosaic Virus RNA with Digoxigenin-Labeled Synthetic Oligo-Deoxyribonucleotide Probes*

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Key words : cucumber mosaic virus, oligo-deoxyribonucleotide probe, digoxigenin-labeled, RNA detection, subgroup-specific detection.

Cucumber mosaic cucumovirus (CMV) has a genome consisting of three plus-sense, single-stranded RNA molecules, designated RNA1, RNA2 and RNA3 in decreasing order of molecular weight, and is divided into subgroups I and II based on serological properties and differences in nucleotide sequence¹⁰.

Many efforts have been made to prevent the enormous damage to agricultural crop production caused by CMV. For example, preinoculation of plants with attenuated CMV was successfully adopted to escape reinfection with more virulent strains¹⁷. A number of studies also describe the establishment of CMV resistance in transgenic plants which express the gene of CMV coat protein, non-structural protein or satellite RNA². The principle of these strategies is based on "cross-protection" of plant viruses. We are now investigating the molecular basis of the phenomenon using various CMV strains. In the course of this study, we need to develop methods that can discriminate the syntheses of subgroups I and II of CMV RNAs in doubly infected cells or leaf tissues.

The identity in nucleotide sequences among RNAs of CMV strains in the same subgroup is over 95%, whereas that between subgroup I and subgroup II is less than 80%¹⁰. Because the 3'-noncoding regions of RNAs 1, 2 and 3 of CMV have highly conserved nucleotide sequences, a DNA or RNA probe complementary to the regions was able to detect all the genomic RNAs at almost the same efficiency³. A comparison of the nucleotide sequences of the 3'-noncoding regions between subgroups I and II revealed a stretch of sequence which was specific for each subgroup. This paper describes the reliability of digoxigenin (DIG)-labeled, synthetic oligo-deoxyribonucleotide probes complementary to the regions for the subgroup-specific detection of CMV RNAs by dot-blot or northern blot analyses.

CMV strains used in this experiment were as follows : subgroup I-CMV-Y¹⁸, CMV-O⁵, both of which belong to subgroup I^{4,6,7,9}, and CMV-m1 that was shown to be of

subgroup I by serological test¹⁶; subgroup II-CMV-m2 and CMV-GT, which were shown to be of subgroup II based on the nucleotide sequences, serological test or symptom observation¹⁶. Viruses were propagated in tobacco plants (*Nicotiana tabacum* L. cv. Xanthi-nc) and purified as described previously¹⁴.

Oligo-deoxyribonucleotides were synthesized by an automated DNA synthesizer. The 5' ends of the DNA were chemically labeled with DIG according to the instructions of the manufacturer (Boehringer Mannheim). Four probes, two for each subgroup, were prepared based essentially on the sequences of the 3'-noncoding regions of CMV-Y RNA^{3,6,7,9} and CMV-m2 RNA^{3,16} as shown in Fig. 1. Probes Y-1 and Y-2 were designed to detect subgroup I CMV. Probe Y-1 was complementary to the sequence of CMV-Y RNA3 having 40 deoxyribonucleotides with a GC content of 52.5%. Probe Y-2 was designed as a degenerate primer of 44 deoxyribonucleotides with a GC content of 47.7-52.3% containing G or A at the positions 8 and 30 from the 5' end, because some variations in the nucleotide sequences at several positions were found among the species of RNA segments and virus isolates belonging to subgroup I. Probes m2-1 and m2-2 for subgroup II are 40 and 45 deoxyribonucleotides long with GC contents of 42.5 and 46.7%, respectively. No nucleotide variations in the regions covered by probes m2-1 and m2-2 were found among RNA3s of subgroup II isolates, CMV-m2, CMV-Q, CMV-Kin and CMV-Trk, when compared using the DDBJ/GenBank/EMBL DNA data base.

Hybridization and detection of RNA on nylon membranes (Hybond-N⁺, Amersham) were carried out as follows. After being baked at 80°C for 2 hr, the blotted membranes were prehybridized in a hybridization buffer containing 5×SSC, 50% formamide, 50 mM phosphate buffer, pH 7.0, 2% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 1 mg/ml yeast total RNA at 45°C for 3 hr. The buffer was replaced with a fresh one containing 20 ng/ml of the DIG-labeled probe.

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Fig. 1. DIG-labeled synthetic oligo-deoxyribonucleotide probes for subgroup-specific detection of CMV RNAs. (A) Comparison of nucleotide sequences near the 3' ends of RNA3s of CMV-Y (subgroup I) and CMV-m2 (subgroup II) where considerable nucleotide changes were found between the two isolates. Identical nucleotides are shaded. Lines with arrows indicate the regions complementary to the respective probes. (B) Structure and nucleotide sequences of the probes. The 5' ends of the probes were chemically labeled with DIG. Nucleotides R at positions 8 and 30 of probe Y-2 indicate A or G.

Fig. 2. Effect of hybridization temperature on the subgroup-specific detection of CMV RNA by dot-blot hybridization using DIG-labeled oligo-deoxyribonucleotide probes. Hybridization was done at 42.5, 45.0 or 47.5°C. Purified virions of CMV-Y and CMV-m2 were dissociated with SDS and dot-blotted onto nylon membranes at various amounts of RNA as indicated at the bottom. The amount of RNA was calculated assuming that the RNA content of CMV was 18.5%.

The membranes were incubated at 42.5, 45.0 or 47.5°C for 16 hr, then washed in 1×SSC containing 0.1% SDS for 20 min followed by washing twice in 0.2×SSC containing 0.1% SDS for 20 min at the fixed temperatures plus 5°C. After washing in maleic buffer (0.1 M maleic acid, pH 7.5, 0.15 M NaCl) containing 0.3% (v/v) Tween 20 for 5 min at room temperature, the membranes were incubated with the maleic buffer containing 1/10 volumes of blocking solution (10% blocking reagent) for 30 min followed by incubation in the same buffer containing 75 mU/ml of sheep anti-DIG Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim) for 30 min. The membranes were then washed twice in the maleic buffer containing 0.3% (v/v) Tween 20 for 15 min. After equilibrating in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM MgCl₂ for 3 min, the membranes were incubated in the same buffer containing 1/2000 volumes of a chemiluminescence reagent, CSPD (Boehringer Mannheim), in plastic hybridization

bags at 37°C for 15 min. The solution was squeezed out of the bags, which were then sealed and exposed to X-ray films for an appropriate time (1 hr to overnight).

To determine the optimum temperature for the hybridization reaction between target RNAs and the probes, dot-blot hybridization was carried out at different temperatures using the four different probes and CMV-Y and CMV-m2 RNAs. Purified virions (1 mg/ml) were dissociated with a final volume of 1% SDS and serially diluted with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, then 1 µl of the solution was spotted onto the nylon membrane (Fig. 2). Probes Y-1 and Y-2 produced clear signals for CMV-Y RNA, but did not for CMV-m2 RNA irrespective of temperature, although probe Y-2 showed approximately 100 times higher sensitivity than probe Y-1. Probe Y-2 could detect less than 10 pg of CMV-Y RNA. Probe m2-1 could specifically detect CMV-m2 RNA although the sensitivity was lower, especially at higher temperatures. In contrast,

Fig. 3. Subgroup-specific detection of CMV RNAs by northern blot hybridization using DIG-labeled oligo-deoxyribonucleotide probes. Total RNA extracted from the tobacco leaves one week after inoculation with CMV-Y or CMV-m2 was electrophoresed in 1% agarose gel, then transferred to nylon membrane. The amount of total RNA per each lane corresponded to that in 7.5 mg of the fresh leaves. Hybridization was done at 45.0°C. Viral RNAs were detected with probe Y-2 or m2-2. Y and m2 indicate the total RNAs from the tobacco leaves infected with CMV-Y and CMV-m2, respectively. H indicates the healthy control.

probe m2-2, which is four bases longer than probe m2-1, exhibited higher sensitivity comparable to that obtained with probe Y-2. Probe m2-2, however, produced higher backgrounds and nonspecific signals at 42.5°C. Based on these results, we decided to use routinely probes Y-2 and m2-2 at 45.0–47.5°C.

Total RNAs of tobacco leaves infected with CMV-Y or CMV-m2 were obtained as described previously¹⁵⁾. The total RNA was electrophoresed in a 1.5% agarose gel under denaturing conditions with 0.66 M formaldehyde, then transferred to a nylon membrane according to standard procedures¹³⁾. Viral RNAs were detected with probe Y-2 or m2-2 as described previously when the hybridization was done at 45.0°C. As shown in Fig. 3, CMV RNAs 1, 2, 3 and 4 were clearly detected in the total RNAs, and each probe hybridized to only CMV RNAs of the corresponding subgroup. The total RNAs from healthy control leaves gave no signals.

To know whether the probes can specifically detect viral RNAs in crude leaf saps, tobacco leaves infected with CMV-Y, CMV-O, CMV-m1, CMV-GT or CMV-m2 were homogenized in five volumes (v/w) of 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM LiCl, 1% SDS and centrifuged at 10,000 rpm for 3 min. Then 1 µl of the supernatant was directly spotted onto the nylon membrane. Viral RNAs hybridized with probe Y-2 or m2-2 at 42.5 or 47.5°C. As shown in Fig. 4, both probes gave specific positive signals without any cross reactions at

Fig. 4. Subgroup-specific detection of CMV RNAs in crude saps of tobacco leaves infected with CMV strains by dot-blot hybridization using DIG-labeled oligo-deoxyribonucleotide probes. Crude saps were prepared by homogenizing the leaves in buffer containing SDS and centrifuging at low speed as described in the text. Hybridization was done at 42.5 or 47.5°C. Tobacco plants were infected with CMV-Y (Y), -O (O), or -m1 (m1) of subgroup I and CMV-GT (GT) or -m2 (m2) of subgroup II. H indicates the crude extracts from the mock-inoculated plants.

47.5°C. In contrast, at 42.5°C, probe m2-2 produced rather strong nonspecific signals and probe Y-2 produced very faint signals for CMV-GT, suggesting that the lower hybridization temperature was also unsuitable for subgroup-specific detection of CMV RNA in crude leaf saps.

Several papers have reported the use of DIG-labeled cDNA or *in vitro* transcribed cRNA probes for the detection of viral RNAs in plants^{1,8,11,12)}. Our results showed that dot-blot and northern blot hybridization using DIG-labeled synthetic oligo-deoxyribonucleotide probes were subgroup specific in detecting RNAs of CMV. We found no cross-reactions between viral RNAs of subgroup I and II and no nonspecific reactions against healthy control samples under suitable conditions for hybridization and exposure to X-ray film. The lower hybridization temperature and prolonged exposure times somewhat decreased the specificity of probe m2-2. Colorimetric detection using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate also gave signals with somewhat lower sensitivity compared to the chemiluminescent one, although the former was more rapid and simple (data not shown).

The length of the probes greatly affected the sensitivity for the detection of CMV RNA. In fact, the extensions of probes Y-1 and m2 with four and five deoxyribonucleotides, respectively, increased the sensitivity approximately 100-fold. Our results showed that short probes of only 44 to 45 nucleotides could detect as little as 10 pg CMV RNA by dot-blot hybridization. This level of sensitivity was comparable with that obtained using

cloned cDNA biotin or ^{32}P -labeled probes for the detection of papaya mosaic potexvirus RNA¹¹⁾.

The long shelf life of the probes and the simplicity, high sensitivity and versatility of our method will greatly facilitate the subgroup-specific detection of CMV RNAs especially in plants or cells that are doubly-infected with CMV isolates of different subgroups.

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和 文 摘 要

高浪洋一・内場隆行・重見知宏・菊原賢次・竹下 稔: DIG 標識合成オリゴデオキシリボヌクレオチドプローブを用いたキュウリモザイクウイルス RNA のサブグループ特異的検出

キュウリモザイクウイルス (CMV) は血清学的性質ならびに塩基配列の違いから subgroup I と II に大別されている。異なる subgroup に属する CMV 分離株に混合感染した感染植物組織内のそれぞれのウイルス RNA を特異的に検出することを目的として、ジゴキシゲニン (DIG) 標識合成プローブを用いた hybridization を試みた。CMV の 3 種のゲノム RNA はほぼ共通した塩基配列の 3' 末端非翻訳領域 (3'NCR) を有することから、1 本のプローブですべての RNA の検出が可能であることが知られているが、3'NCR の一部に subgroup 間で塩基配列が大きく異なる領域が存在する。その領域に相補的な DIG 標識合成オリゴデオキシリボヌクレオチドをそれぞれプローブとして準備し、nylon membrane 上で dot-blot および northern blot hybridization を行った。alkaline phosphatase 標識 DIG 抗体および化学発光基質を用いた場合、dot-blot で 10 pg の CMV RNA が検出可能であった。また、northern blot hybridization においては各 subgroup の RNA1~4 のすべてが特異的に検出された。さらに、感染粗汁液を直接プロットした場合でも subgroup 特異的な CMV RNA の検出が可能であった。本法は、標識プローブの長期安定性、操作の簡便性ならびに放射能を用いる方法に比肩し得る十分な検出感度などの利点を有し、CMV RNA の subgroup 特異的検出に有用と思われる。

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