식물학회지 제35권 제3호
Korean J. Bot. 35(3) : 253~257

Molecular Cloning of cDNA for Garlic Mosaic Virus Genome

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마늘 모자이크 바이러스 게놈에 대한 cDNA의 클로닝

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ABSTRACT

Potyvirus group is the largest group among plant virus groups and damages severely plant hosts upon infection. In order to investigate the mechanism by which potyviruses induce disease in plants, a cDNA clone 29-6 which is considered to be a cDNA clone for garlic mosaic virus (GMV) was isolated. It did not hybridize to garlic latent virus genome, which is one of two major garlic viruses. Northern blot analysis shows that the genome size of garlic mosaic virus was about 9 kb. Clone 29-6 strongly hybridizes to poly(A) RNA isolated from garlic leaves, suggesting that GMV RNA is polyadenylated as other potyviruses. Nucleotide sequence analysis of cDNA clones overlapping with clone 29-6 showed that garlic plants are infected with various strains of garlic mosaic virus which are closely related to each other.

INTRODUCTION

Most of the garlic plants cultivated throughout the world are thought to be infected with viruses which give rise to mosaic symptom on leaves and reduction of yield (La, 1973; Ahlawat, 1974; Chung and Chang, 1979; Lee *et al.*, 1979; Grimsley *et al.*, 1987). The wide occurrence of the virus disease of garlic is due to the facts that there are no practical control for the disease, and that the production of garlic has solely depended on vegetative propagation. Although there have been some attempts to produce the virus-free seed garlic by tissue culture techniques, viruses were often not successfully eliminated even from tissue cultured garlic (Havranek, 1973; Kehr and Schaeffer, 1976; Chang *et al.*, 1980; Lee, 1981). Virus particles are found in the cytoplasm of apical meristem domes of garlic cultivars. The analysis of transverse serial ultrathin sections of apical meristems of garlic bulb of donor plants and plantlets cultured from excised meristems, confirmed the presence of virus in the meristematic regions. These observations provide an example for the difficulties encountered in obtaining virus-free garlic plantlets by apical meristem culture (Chang *et al.*, 1988). In most cases, moreover, virus-free garlic would be infected with virus again when planted in the field.

Two sap-transmissible elongated viruses from garlic plants were described and named garlic latent virus (GLV) and garlic mosaic virus (GMV) based on the symptoms produced in infected garlic plants and the observation by electron microscope (Lee *et al.*, 1979; Chang *et al.*, 1980, 1988; La and Choi, 1987). GLV seems to be a carlavirus with around 650-700 nm long flexous rod shape and GMV seems to be a potyvirus of about 750-800 nm long on electron micrograph. Mixed infection of GLV and GMV was found in most of the garlic plants showing mosaic symptoms collected from various parts of Japan and Korea (Lee *et al.*, 1979; Chang *et al.*, 1980, 1988; La and Choi, 1987).

Symptoms due to the infection of GMV was mosaic in garlic plants, and local necrotic lesions in *Gomphrena globosa*, whereas latent infection was detected in the inoculated leaves of *C. amaranticolor*, *C. quinoa* and *T. expansa*. GMV, garlic yellow streak virus (GYSV), leek yellow stripe virus (LYSV) and onion yellow dwarf virus (OYDV) are known as potyviruses occurring in vegetable allium plants (Noda and Inouye, 1989).

In the previous experiments we have isolated several cDNA clones for garlic viruses and classified these clones into 4 different groups on the basis of their cross hybridization (Choi *et al.*, 1991). The second largest group was identified to be cDNA clones for GLV which is one of the two most abundant garlic viruses by Northern blot analysis with genome of purified GLV (Choi *et al.*, 1991). The most frequently encountered group was concluded to be cDNA clones for GMV in this report. Molecular characterization of GMV genome and its cDNA clones is reported.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in these experiments are *E. coli* MC1061 and JM101 for transformation and plasmid conservation, and *E. coli* Y1090(pMC 9) for λgt11 phage host.

Enzymes and chemicals. Restriction enzymes were purchased from KOSCO and Promega. Nitrocellulose paper was from Schleicher & Schuell. Other chemicals were obtained from Sigma Chemical Co.

Virus preparation. Garlic virus was isolated from the mosaic diseased garlic leaves by the procedure described by Langenberg (1973). Virus-infected garlic leaves were ground in cold TAC extraction buffer (0.1 M Tris, pH 7.2, 0.05 M citric acid, 0.8% polyvinyl pyrrolidone and 0.2% 2-mercaptoethanol) (2.5 ml/g leaf tissue) with sea sand in pre-chilled mortar. The extract was transferred into a centrifuge tube and 1 ml of 2.0 M $CaCl_2$ and then 2 ml of 2.0 M K_2HPO_4 were added for each 50 ml extract with stirring. It was centrifuged for 10 min at 10,000 rpm with JA-20 rotor at 4°C. The supernatant was recovered, and Triton X-100 and polyethylene glycol MW 6,000 (PEG 6,000), were added to make final concentrations of 0.5% and 6%, respectively. The solution was stirred for 1 h at 4°C and stood for at least 1 h at 4°C. The precipitate formed was centrifuged for 20 min at 10,000 rpm with JA-20 rotor at 4°C. Pellets were resuspended in cold TACm buffer (0.01 M Tris, pH 7.2; 0.005 M citric acid; 0.01 M 2-mercaptoethanol; 0.1% Triton X-100) to give a final volume one-twentieth of the extract. The resulting suspension was centrifuged for 10 min at 10,000 rpm with JA-20 rotor to remove insoluble matter. The supernatant was overlaid on 30% sucrose dissolved in TACm buffer and centrifuged for 1 h at 68,000×g with fixed angle rotor at 4°C. The pellet was dissolved in TACm buffer and transferred into an Eppendorf tube. Insoluble matter was removed by centrifugation for 1 min at 10,000 rpm. The supernatant was overlayered on linear 10~40% sucrose density gradient and centrifuged for 2 h at 55,000×g with aluminium swing-out rotor at 4°C. Centrifuged fractions were scanned photometrically at 260 nm. Virus genomic RNA was isolated by the phenol extraction method (Sambrook *et al.*, 1989).

Nucleotide sequencing. Nucleotide sequencing was carried out after serial deletion construct preparation with Exonuclease III. Unidirectional digestion was carried out by the procedure developed by Henikoff (1984). Nucleotide sequencing was carried out in plasmid pUC18(19) (Yanisch-Perron *et al.*, 1985) by the dideoxynucleotide chain termination method of Sanger *et al.* (1977). Universal M13 primers for reverse and forward reaction were used and the reaction products were analyzed by 6 M urea-polyacrylamide gel electrophoresis.

Recombinant DNA techniques. Plasmid DNA was isolated as previously described by Sambrook *et al.* (1989). Southern and Northern blot analysis, nick translation and hybridization were carried out by the procedure of Sambrook *et al.* (1989). Other recombinant DNA manipulation was carried out by standard protocols of Sambrook *et al.* (1989).

RESULTS AND DISCUSSION

Genome analysis for garlic mosaic virus. In the previous experiments we have isolated several cDNA clones for garlic viruses and classified these clones into 4 different groups on the basis of cross hybridization (Choi *et al.*, 1991). The second largest group was identified to be cDNA clones for GLV by Northern blot analysis with genome of purified GLV (Choi *et al.*, 1991). Molecular characterization of cDNA clones of the most largest group is reported in this paper.

To investigate the size of virus genome and the RNA transcript corresponding to the isolated cDNA clones of the largest group, Northern blot analysis of garlic virus RNA isolated from mosaic discased garlic was carried out with the cDNA clone 29-6 of garlic virus as a molecular probe. Garlic virus RNA preparation showed strong

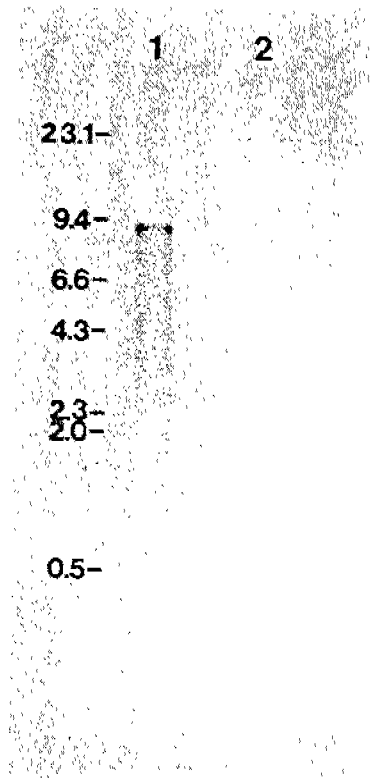

Fig. 1. Northern blot analysis of garlic mosaic virus. RNA was separated in a 0.8% formaldehyde agarose gel and transferred onto nylon membrane and hybridized with random primer-extended inserts of clone 29-6. Lane 1: garlic virus RNA. Lane 2: garlic latent virus RNA.

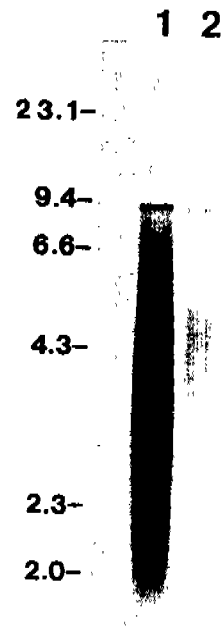

Fig. 2. Northern blot analysis of garlic poly(A)$^+$ RNA. RNA was separated in a 0.8% formaldehyde agarose gel and transferred onto nylon membrane and hybridized with random primer-extended inserts of clone 29-6. Lane 1: garlic leaf poly(A)$^+$ RNA. Lane 2: garlic leaf total RNA.

signal at the position of around 9.0 kb (Fig. 1). It corresponded to the major band of RNA on formaldehyde agarose gel stained with ethidium bromide. When the same amount of GLV RNA which is one of the two major viruses of garlic was loaded side by side (Choi *et al.*, 1992), the clone 29-6 did not hybridized to it at all, if any, suggesting that the clone 29-6 is a clone for one of garlic viruses whose genome size is around 9.0 kb but not a cDNA clone for GLV of similar size. Potyvirus is known to be usually 9-10 kb long flexous rod shape. When the same blot was washed off and reprobed with a cDNA clone for GLV, GLV lane showed stronger signal, suggesting the presence of virus RNA in both lanes and reflect relative amount of virus genome present in garlic (data not shown).

Polyadenylation of the RNA genome corresponding to the cDNA clone 29-6 was investigated by Northern blot analysis of poly(A)$^+$ RNA isolated by oligo(dT) cellulose chromatography (Fig. 2). Strong signal at the size of 9.0 kb was prominent suggesting that viral RNA for clone 29-6 is polyadenylated as other potyviruses such as tobacco vein mottling virus (Domier *et al.*, 1986). Members of the potyvirus group have a genome consisting of an infectious plus-sense single-stranded RNA molecule of approximately 10 kb, which is polyadenylated at its 3′ terminus and covalently linked to a small viral protein (termed VPg) at its 5′end (Dougherty and Carrington, 1988).

These results altogether strongly suggest that clone 29-6 may be a cDNA clone for GMV rather than GLV. Further identification with purified GMV is under progress.

Nucleotide sequence analysis of garlic virus cDNA clones. The clone 29-6 was employed as a probe to isolate other cDNA clones for the genome of GMV. Furthrmore, chromosomal walking was carried out by subse-

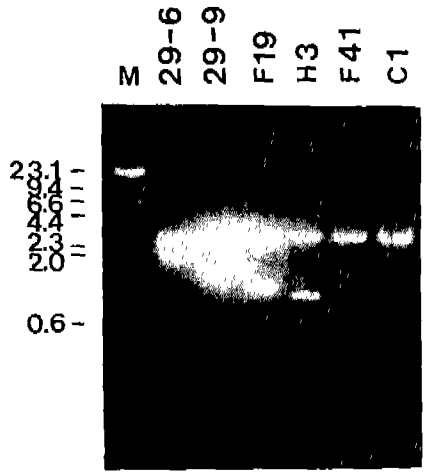

Fig. 3. Electrophoresis in a 1% agrose gel of *Eco*RI-digested DNAs of clone 29-6 and its related clones for garlic mosaic virus. The names of the clones are indicated on the top and the size markers (M) are λDNA digested with *Hin*dIII.

quent screening of garlic virus cDNA library (Choi *et al.*, 1991) using isolated cDNAs as probes. So far, 5 additional clones were isolated as shown in Fig. 3. The complete or partial nucleotide sequences of these clones were also determined. The extent of overlapping of each of these clones is shown in Fig. 4 as determined on the basis of their nucleotide sequences. They covered the 3 kb of the GMV genome. The nucleotide sequences of part of clones 29-6, F19, F41, and C1 were compared as shown in Fig. 5. Interestingly, they were not identical at the overlapped region. Clone 29-6 was almost identical to clone F41, differing in 2 among 200 nucleotides compared. However, clone 29-6 was different in 34 among 200 nucleotides to clones F19 and C1, both of which were almost identical except in 7 nucleotides. The overlapping region between clone 29-6 and 29-9 (Fig. 4) also showed about 20% difference in the nucleotide sequence. Most of base changes were transitions. This implys that garlic plants are infected with various GMV strains. This may be due to the facts that garlic plants have been cultivated asexually for over 2000 years in Korea and that RNA genome is less stable than DNA genome, accumulating more mutations in the genome. Electron microgram of virus particles purified from infected garlic leaves also revealed that garlic viruses are various in length ranging from 200 to 2000 nm, although most are in range of 600 and 900 nm (Choi *et al.*, 1992).

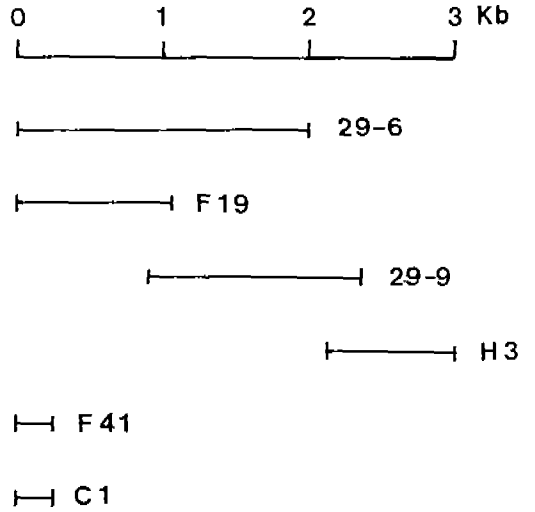

Fig. 4. Relationship among the clones overlapping with clone 29-6 as determined by nucleotide sequencing.

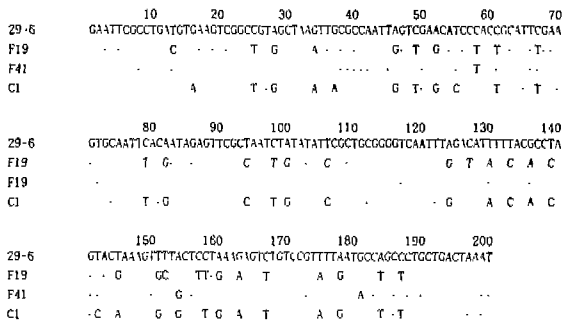

Fig. 5. Comparison of nucleotide sequences of part of clones 29-6, F19, F41 and C1. Nucleotide seqeunces shared by the four clones are indicated by dots.

Complete nucleotide sequence data exist for four different potyviruses and predict a single open reading frame (ORF) with the potential to encode a polyprotein of molecular weight being approximately 340,000 Da (340 kDa). This large precursor polyprotein is processed co- and post-translationally to form at least eight polypeptides (Dougherty and Carrington, 1988). Comparison of the nucleotide sequence of the clone 29-6 to those of potyviruses showed little homology. Therefore, the cDNA clone 29-6 seemed to correspond to less conserved sequence region of these potyviruses. The fact that GMV is the most abundant virus in mosaic diseased garlic increases the possibility to obtain a right clone for GMV. Nucleotide sequencing of full-length genome of GMV is under

progress.

As is in this report, isolation of total virus particles from mixed infected plant and construction of cDNA library from them could provide a good chance to study molecular structure of a specific virus.

적 요

Potyvirus group에 속하는 것으로 알려진 마늘 모자이크 바이러스 (GMV)가 식물에서 병을 유도하는 메카니즘을 이해하기 위하여, 마늘에 존재하는 GMV에 대한 cDNA clone인 clone 29-6을 분리하였다. Northern blot 분석에 의해 이 바이러스의 genome size는 약 9 kb이고, 또한 clone 29-6은 GLV genome과 유사성이 없었으며, 마늘 잎으로부터 분리된 poly(A)$^+$ RNA와 강하게 hybridization 되었다. 이러한 사실은 이 cDNA clone이 마늘에 감염하여 모자이크 병반을 유도하는 GMV에 대한 cDNA clone 중의 하나인 것으로 생각되었다. Clone 29-6을 probe으로 사용하여 마늘 바이러스의 cDNA 은행을 탐색하여 이와 중첩되는 clone을 선별하여 염기서열을 결정한 결과 이들의 염기서열이 중첩부위에서 서로 일치하지 않았는데 이는 마늘은 여러 형태의 GMV에 의해 감염되어 있음을 암시한다.

ACKNOWLEDGMENT

The present investigation was supported by the grant from Genetic Engineering Program (1990), Ministry of Education, Republic of Korea.

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(Received July 14, 1992)