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Molecular cloning of cDNAs for Korean garlic viruses

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Abstract : To understand the molecular structure and pathogenesis mechanism of Korean garlic viruses (GV), virus particles were isolated from field-grown garlic leaves and RNA genome was isolated from them. It was used for constructing cDNA library for GV. Several cDNA clones for GV were isolated and classified into 4 different groups on the basis of cross Southern hybridization. Northern blot analysis of GV RNA with one of these cDNA clones shows that the clone is a cDNA for GV RNA(Received July 4, 1993; accepted July 28, 1993).

Introduction

Garlic (*Allium sativum* L.) is an important vegetable crop for the Korean people and has long been cultivated extensively in Korea. Currently garlic mosaic disease is considered to be the most important disease of garlic in Korea. Two sap-transmissible filamentous viruses from garlic plants were described and named as garlic latent virus (GLV) and garlic mosaic virus (GMV) based on the microscopic observation and the symptoms produced in infected garlic plants.[1] Symptoms due to the infection of GMV was mosaic in garlic plants. Garlic plants inoculated with GLV produced visible symptoms at early stage of infection but less prominent at later stage.[1-3] Particles of GMV were flexuous rods about 750 nm long, as is most of the potyvirus pinwheel-type cytoplasmic inclusions found in the infected garlic plant cells.[1] Particles of GLV were flexuous rods, 650~700 nm long, and existed as either randomly or small aggregates in the cytoplasm of the infected plant cells as is most of the carlavirus. However, mixed infection of GLV and GMV were found in most of the garlic plants showing mosaic symptoms collected from various parts of Korea and Japan.[1] Present evidences indicate that most, if not all, unselected commercial garlic cultivars contain a complex of two or more viruses.[4-6] But the identity of the individual viruses contained in each complex is variable, particularly in materials from diverse geographical locations. Garlic mosaic virus (GMV), garlic yellow streak virus, leek yellow stripe virus and onion yellow dwarf virus are known as potyviruses occurring in vegetable Allium plants.[7] Therefore, the identification of viruses infecting garlic is complex and the related literatures are not clear in this aspect.[6]

To understand the molecular structure and identities of garlic viruses, molecular cloning of cDNAs for viral genome was attempted. Virus particles were isolated from field-grown garlic leaves and a cDNA library was constructed. Molecular evidences are described for the cDNA cloning of garlic viruses.

Materials and Methods

Bacterial strains

Bacterial strains used in this experiments are *E. coli* MC1061 [F^-, *araD139*, $\triangle$(*ara, Leu*)*7696*, $\triangle$*lacY74*, $galU^-$, $galK^-$, $hsdR^-$, *strA*] for transformation and plasmid conservation, and *E. coli* Y1090 [*supF*, *hsdR*, *araD 139*, $\triangle$*lon*, $\triangle$*lacU169*, *rpsL*, *trpC22*::*Tn10* (*tetr*), *pMC9*]

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for λgt11 phage host.

Enzymes and Chemicals

E. coli DNA polymerase I, Klenow fragment and T4 DNA ligase were purchased from New England Biolabs. *Eco*RI, *Hin*dIII, *Sal*I, *Bam*HI and *Pst*I restriction enzymes were from KOSCO. [α-^{32}P]dATP, [γ-^{32}P]ATP, lambda gt11 vector DNA and in vitro packaging system were from Amersham. DEAE-cellulose (DE-52) was from Whatmann and nylon membrane from Du Pont. Other chemicals were purchased from Sigma Chemical Co.

Garlic samples

Garlic (*Allium sativum* L.) samples used in this experiment were garlic leaves grown at the experimental farm of Seoul National University, and part of them were kept at −70℃.

Virus preparation

Garlic virus particles were isolated from the virus-infected garlic leaves by the procedure described by Langenberg[8] and La.[9] Virus-infected garlic leaves were ground in cold 0.1 M TAC extraction buffer (2.5 m*l*/g leaf tissue) with sea sand in a pre-chilled mortar. TAC extraction buffer consists of 0.1 M Tris, pH 7.2, 0.05 M citric acid, 0.8% polyvinyl pyrrolidone, and 0.2% 2-mercaptoethanol. One m*l* of 2.0 M $CaCl_2$ and 2 m*l* of 2.0 M K_2HPO_4 were added for each 50 m*l* extract with stirring and the extract was centrifuged for 10 min at 10,000 rpm with JA-20 rotor at 4℃. Triton X-100 and polyethylene glycol (MW 6,000) were added to the supernatant to final concentrations of 0.5% and 6%, respectively. The solution was stirred for 1 hr and stood for at least 1 hr at 4℃ and centrifuged for 20 min at 10,000 rpm. The pellets were resuspended in cold TACm buffer (10 mM Tris, pH 7.2; 5 mM citric acid; 10 mM 2-mercaptoethanol; 0.1% Triton X-100) to give a final volume of one-twentieth of the extract. The suspension was centrifuged for 10 min at 10,000 rpm to remove insoluble matter and the supernatant was overlaid on 30% sucrose dissolved in TACm buffer. After centrifugation for 1 hr at 68,000 *g* in fixed angle rotor at 4℃, the pellet was dissolved in TACm buffer and insoluble matter was removed by centrifugation for 1 min at 10,000 rpm. The supernatant was overlaid on linear 10～40% sucrose density gradient and centrifuged for 2 hrs at 55,000 *g* with swing-out rotor (RPS55T-2) at 4℃. The 0.4 m*l* fractions from sucrose gradient were scanned photometrically at 260 nm and analyzed by SDS-PAGE.

SDS-polyacrylamide gel elecrtrophoresis

Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis.[10] The separating gel was prepared from a stock of 33.5% acrylamide and 0.3% *N,N'*-bisacrylamide to a final concentration of 12.5% acrylamide. The separating gel buffer contained 0.38 M Tris-HCl, pH 9.1. The stacking gel was prepared from a stock of 30% acrylamide and 0.44% *N,N'*-bisacrylamide to a final acrylamide concentration of 4% in 0.125 M Tris-HCl, pH 6.8. Both gel contained 0.1% SDS and were polymerized with ammonium persulfate and *N,N,N',N'*-tetramethylenediamine. The electrode tank buffer was 25 mM Tris, 192 mM glycine containing 0.1% SDS. Samples were prepared by boiling for 3 min in 0.125 M Tris-HCl (pH 6.8) buffer containing 5% 2-mercaptoethanol, 1% SDS, 10% glycerol and bromophenol blue.

Construction and screening of garlic virus cDNA library

cDNA library was constructed in λgt11 by the method of Gubler and Hoffman[11] with minor modification. Five μg of garlic virus RNA isolated from virus-infected garlic leaves was used as a template. Mixture of oligo (dT) and random hexamer were used as primers. Double stranded cDNA was ligated into λgt11 vector using a ratio of insert DNA : vector DNA of 1 : 20 by mass and packaged in vitro. Screening was carried out with the radio-labeled first strand cDNA for garlic virus.

Nucleic acid preparation and recombinant DNA techniques

Recombinant phage DNA was isolated by DEAE-cellulose chromatography.[12] Plasmid DNA was isolated as described by Sambrook *et al.*.[13] Southern blot analysis was carried out according to the procedure of Sambrook *et al.*[13] and Northern blot was carried out by

the procedure of Kroczek and Siebert.[14] Nick translation and hybridization were carried out by the standard procedure of Sambrook *et al.*[13]

Results and Discussion

To study molecular structure of GV, the virus particle was isolated from virus-infected garlic leaves by Langenberg method[8] with minor modification. It was purified by 10~40% sucrose density gradient sedimentation and analyzed by 12.5% SDS-polyacrylamide gel electrophoresis (Fig. 1). Even though it was rather broad, the sixth fraction out of ten fractions from the top showed the highest intensity of proteins. At least five protein bands were resolved and the molecular weight of the proteins were about 23,000~34,000. These proteins could be regarded as structural proteins of garlic viruses. Therefore, the virus preparation can be considered to contain at least five different kinds of viruses, if each virus is made up of a single kind of coat protein. It also suggests that field grown garlic plants are indeed infected with multiple species of viruses.

To isolate the nucleic acid from GV, the peak fraction was treated with SDS, EDTA, and Proteinase K and analyzed by 1.2% formaldehyde agarose gel electrophoresis (Fig. 2, lanes 1, 2). The major band of 8.2 kb was the same as the genome size predicted from the size of rod-shaped GV particles of about 700 nm. When the nucleic acid was treated with RNase A (50 μg/50 μl for 30 min at 37℃), the major band disappeared, which suggesting that GV is a RNA virus (Fig. 2, lane 3).

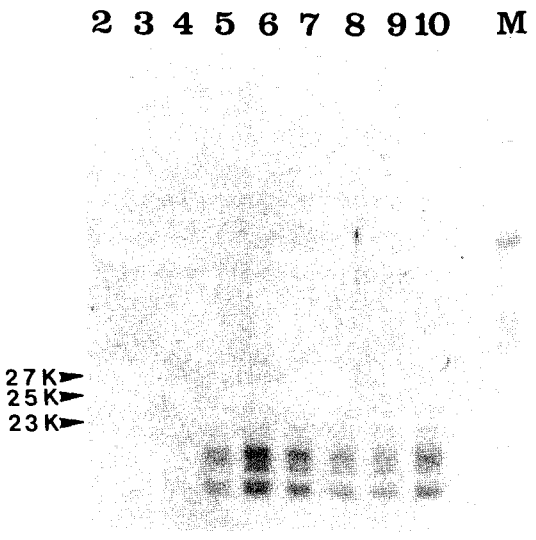

Fig. 1. SDS-PAGE analysis of garlic virus prepared from field-grown garlic leaves. Protein samples were analyzed by 12.5% SDS-PAGE and stained with Coomassie Blue.
Lanes 2~10: fractions from 10~40% sucrose density gradient from the top

To isolate cDNA clones for the GV, garlic virus cDNA library was constructed into λgt11 vector. The titer of this library was 7.7×10^5 pfu/m*l* and 70% of them was recombinant.

Eleven cDNA clones from 0.2 kb to 1.3 kb in size were isolated by hybridization with the first strand cDNA as a probe (Fig. 3). To figure out the relationship between these clones, cross Southern hybridization was attempted with the clones 28, 29, 6 and 18 as molecular probes (Table 1). In case of the clone 28 as a probe, clones 16 and 6 were hybridized. Similarly the clone 29 was hybridized to the clone 59 and the clone 18 hybridized to clones 3 and 12. The clone 28, however, was not hybridized to the clones 29 and 59, and the clone 29 was not hybridized to clones 28, 16 and 6, either. As a result, these clones were divided into three groups which do not hybridize each other. The fourth group was made up with clones which was not cross-hybridized to any other clones. This result suggests that each group could be cDNA clones of different kind of virus. Also it is possible that they are different part of one virus genome.

To obtain a cDNA clone spanning virus full genome,

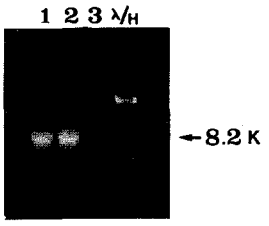

Fig. 2. Size analysis of garlic virus RNA. RNA was analyzed by 1.2% formaldehyde agarose gel electrophoresis and stained with ethidium bromide. Lanes 1 and 2 are from two different virus preparation. Virus genome of lane 1 was treated with RNase (50 μg/50 μl for 30 min at 37℃, and analyzed in lane 3. λ/*Hin*dIII size markers are shown.