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Determination of Nucleotide Sequences of cDNA from Cucumber Mosaic Virus-As RNA4

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As계 오이 모자이크 바이러스 RNA4의 염기서열 결정

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ABSTRACT : The cDNA of full-length RNA4 (1,043 nt's) of cucumber mosaic virus-As strain (CMV-As) was constructed and its complete nucleotide sequences were determined and characterized. It contains 5'-leader region of 74 nt's, viral coat protein gene region of 657 nt's and 3' noncoding region of 312 nt's. Although nucleotide sequences of the coding region are mostly invariable among different strains, those of 5'-leader and 3'-untranslated regions are diverse. In particular, 61 nt's at the 3'-end region (959 to 1,019) in other strains are very similar among one another, but remarkably different from that of CMV-As. However, the secondary tRNA-like pseudoknot structure can be folded at its 3'-end region even though there are considerable sequence variations. Comparison of the nucleotide sequence of CMV-As showed that the highest degree of identity was found in CMV-I17F (91.9 %) whereas it is much lower in S-type strain, CMV-Q (71.1%). Based upon the data from sequence analysis and the presence of an internal *Eco*RI restriction site, CMV-As strain is classified into WT-type of CMV.

***Key words* : CMV-As, RNA4, nucleotide sequence, WT-type, tRNA-like pseudoknot, classification.**

Cucumber mosaic virus (CMV) has a very broad host range. It infects diverse groups of plants including vegetables such as cucumbers, tomatoes, peppers, etc. (9, 10). Symptoms expressed on infected plants usually display from mild to strong mosaic phenomena and stunting with or without leaf deformation. CMV, the type member of the cucumovirus group, forms isometric particles with a tripartite, single-stranded RNA genome of messenger polarity. It consists of three RNA species which are called as RNA1 (3.4 kb), RNA 2 (3.1 kb) and RNA3 (2.2 kb), respectively, in the order of decreasing molecular weight (8, 13, 20, 28). It also contains an additional RNA species, designated as RNA4 (1.0 kb) which is a subgenomic messenger RNA of 3' half of RNA3 for the synthesis of viral coat protein (CP). Both RNA1 and RNA2 have been reported to encode polypeptides responsible for virus replication; RNA1 codes for helicase-like protein and RNA2 for RNA-dependent RNA polymerase (RdRp), respectively. RNA3 presumably encodes cell-to-cell movement protein (also called as 3A protein) and CP. However, some strains of CMV contain an additional small virus-dependent satellite RNA, RNA5 (also called as CARNA5, CMV-associated RNA5), which alters symptoms of the disease (5, 12, 14, 29). Interestingly, the 3' ends of these RNAs except RNA5 have been found to form tRNA-like pseudoknot structure whose functional significance has not yet been elucidated. But some reports suggested that it should be an important recognition site for viral replicase holoenzyme (2, 15, 22, 31), or at least crucial for the expression of symptoms by viral infection. In addition, these RNAs of CMV can be aminoacylated with tyrosine by its cognate tRNA synthetase (11).

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Currently, more than 60 strains of CMV have been reported and classified into two major subgroups; WT and S (or I and II, respectively) (1, 24). They have been grouped in terms of nucleic acid hybridization studies (21), but it may not be sufficient to suggest the phylogenetic relatedness of the genomes from different strains in the same subgroups.

The complete nucleotide sequence of CMV-Q strain has been reported (4). But it is a representative of S subgroup consisting of only 3 members, contrary to 17 members in the WT subgroup (24). Extensive informations of genomic sequences have been generated mostly for the members of WT group as a portion from the whole genome. Nucleotide sequences of genomes from the following strains have been elucidated; RNA3 from strains Q (4), O (7), C, WL (21), Y (16), M and Fny (18), and RNA4 from strains D (3) and I17F (17). Because of the limited sequence information mentioned above, additional sequence data for more genomic RNAs are, therefore, necessary to characterize genome organization, the degree of nucleotide sequence variation, and evolutionary relationships among them.

This study was focused on aster (As) strain of CMV, the Korean isolate, purified from *Aster yomena* Makino (19). And we cloned the full length cDNA from RNA4 of CMV-As and determined its the nucleotide sequence. Its nucleotide sequence was compared to those published for other CMV strains.

MATERIALS AND METHODS

Purification of virus and RNA from CMV-As. Cucumber mosaic virus isolated from *Aster yomena* (CMV-As) was multiplied in tobacco plant (*Nicotiana tabacum* cv. Xanthi-nc) (19). One kilogram of tobacco leaves were homogenized in 2 volumes (w/v) of 0.5 M sodium citrate buffer, pH 6.5, containing 10 mM EDTA and 0.1% thioglycolic acid and an equal volume of chloroform at 4°C before centrifugation at 5,500×g. To precipitate virus particles, polyethylene glycol (PEG) 8,000 and NaCl were added to the supernatant at 4°C to final concentrations of 8% and 0.1 M, respectively. Further purification was followed by standard methods (28) with slight modifications. An average yield of CMV particles isolated was calculated as 8 mg/kg tissues. Genomic RNAs of CMV were extracted from these virus particles by SDS-phenol extraction procedure (28).

Synthesis of double-stranded cDNA. The polyadenylation reaction was carried out using the procedure of Smith *et al.* (30). Thirty-five micrograms of CMV-As RNA and 250 units of *E. coli* poly A polymerase (BRL) per milliliter were incubated at 37°C for 10 minutes in a buffer containing 50 mM Tris-Cl, pH 7.9, 25 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 10 mM DTT, 0.5 mg/ml BSA and 0.1 mM ATP before precipitation with ethanol. The polyadenylated RNAs were then purified through oligo-dT cellulose column chromatography (Pharmacia LKB). The synthesis of double-stranded cDNA from CMV-As RNA was performed using RiboClone cDNA synthesis system (Promega).

Cloning of cDNA. The blunt-ended cDNA synthesized was purified through Sephadex G-50 column (26). It was then ligated with 10 picomoles of *Eco*RI adaptor (Amersham Life Science). cDNA molecule with *Eco*RI adaptor was size-fractionated by Sepharose CL-4B (Size-500, Phamacia) column chromatography followed by phosphorylation with T4 polynucleotide kinase. The cDNA pool was then ligated into pT7T3 19U (Phamacia) and transferred to *E. coli* strain NM522.

The conventional recombinant DNA manipulations were performed according to the methods described in Sambrook *et al.* (26). DNA sequences of cloned cDNA were determined by dideoxy chain termination method (27) using Sequenase 2.0 kit (USB) and 17-mer M13/pUC or T7 reverse primer.

Slot-blot hybridization. To identify whether pAS 66 contains cDNA insert from RNA4 of CMV-As, slot-blot hybridization was carried out (26). RNAs or DNAs were denatured in a buffer containing 50% deionized formamide, 6.5% formaldehyde and 0.5X SSC (20X SSC: 3 M NaCl and 0.3 M Na-citrate, pH 7.0) at 65°C for 15 minutes. Following the addition of 61 μl of 20X SSC, nucleic acids were applied to nylon membrane by slot-blot kit (Hoffer Scientific Instruments, HSI). The membrane was then washed in 10X SSC and baked at 80°C for 2 min. Prehybridization was carried out in a 18 ml of solution containing 50% formamide, 5X SSC, 0.14% SDS, 5X Denhardt's solution, 25 mM Na-phosphate (pH 6.5) and 100 μg/ml salmon sperm DNA at 42°C for 4 hrs. Hybridization was also carried out in a solution additionally containing 5% dextran sulfate and the ^{35}S-labelled DNA insert probe at 42°C for 16 hrs. The membrane was then washed 3 times for 15 min each in 0.2X SSC containing 0.1% SDS, dried and exposed to X-ray film overnight at room temperature.

RESULTS AND DISCUSSION

Isolation of cDNA from CMV-As RNA4. The plasmid containing about 1 kb DNA insert was isolated from the plasmid library and designated as pAS66. Because the *Eco*RI adaptor molecule (Amersham) additionally contains 3 different restriction sites, the clone pAS66 was digested with either *Eco*RI, *Bam*HI or *Kpn*I. As shown in Fig. 1, pAS66 possesses 1 kb cDNA insert presumably originated from RNA4. Also, it was shown to contain an additional *Eco*RI restriction site inside of the 1 kb cDNA insert. This observation is in accordance with a recent report about the classification method (1). Namely, the existence of *Eco*RI recognition site in CMV RNA4 cDNA sequences was used for classifying CMV isolates in a simple way.

The *Bam*HI fragment of pAS66 (i.e. 1 kb insert) was used as a probe for the hybridization to CMV-As RNAs and to pCMV7 DNA (from DSM: Deutsche Sammlung von Mikroorganismen) raised from CMV-D RNA4. However, the probe was not hybridized to pCMV1- cDNA clone from CMV-D RNA1 (from DSM), and ORSV (odontoglossum ringspot virus) RNA as a negative control (Fig. 2). Both CMV-As total RNAs and pCMV7 DNA were hybridized to the probe. Therefore, pAS66 was determined to contain the cDNA insert derived from RNA4. Taken together with this hybridization data and the presence of *Eco*RI site in CMV RNA cDNA, CMV-As can be assigned to subgroup II.

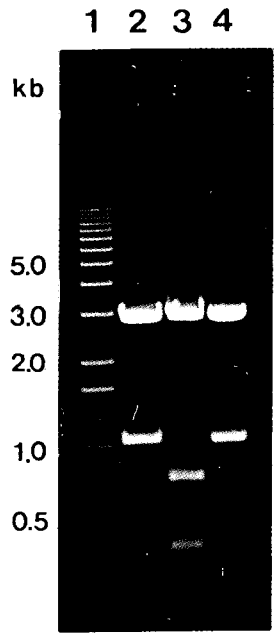

Fig. 1. Fractionation of pAS66 plasmid DNA containing CMV-As RNA4 cDNA insert on 1% agarose gel. pAS66 DNA was digested with either *Bam*HI (lane 2),. *Eco*RI (lane 3) or *Kpn*I (lane 4). Lane 1; 1 kb ladder (BRL).

Nucleotide sequenses of cDNA from CMV-As RNA4. The cDNA insert for RNA4 in pAS66 was digested with either *Bam*HI, *Hin*cII, *Eco*RV or *Eco*RI to produce suitable fragments for subcloning and sequencing. Subsequently these fragments were cloned into either pUC118 or pUC119 and their sequences were analyzed. Then, the full-length cDNA sequences from CMV-As RNA4 of 1,043 bp in size has been completely identified. As shown in Fig. 3, CMV-As RNA4 consists of 3 regions-74 nucleotides of the 5'-leader region, 657 nucleotides encoding a viral coat protein and 312 nucleotides of the 3' noncoding region. As compared with CMV-Y (16), -O (7), -Q (4), -D (3), -I17F (17), -Fny and -M (18), the CMV-As RNA4 has nucleotide sequence identities ranging from 71.1% to 91.9%, whereas it is a little higher in amino acid sequences for coding region ranging from 72.0% to 97.2 % (Table 1). It indicated that the nucleotide sequence of the coding region is invariable, but those of the 5'-leader and the 3'-untranslated region are not. However, the heptanucleotide sequences reported for the 18S ribosomal RNA binding site are conserved throughout different strains except for strain Q (Fig. 3) (32). Al-

Fig. 2. Autoradiogram of slot-blot hybridization of cDNA clones and viral RNA probed by *Bam*HI cDNA insert from pAS66 plasmid DNA. Slots 1 to 3, 1 μg, 100 ng and 10 ng each of pAS66 DNA; slot 5, 1 μg of poly A CMV-As RNA; slot 10, 1 μg of pT7T3 19U; slot 11, buffer only; slots 11 to 13, 1 μg, 100 ng and 10 ng each of pCMV1, cDNA from CMV-D RNA1; slot 18, buffer only; slot 20 1 μg of ORSV (odontoglossum ringspot virus) RNA; slots 4, 9, 14 and 19, blanks.

though 61 nucleotides at the 3'-end region (959 to 1,019) are mostly invariable among other strains, it is extensively diversified in the CMV-As (Fig. 3). In spite of this variation, it is presumed that the secondary tRNA-like structure is conserved at its 3' end (Fig. 4). Therefore, the phylogenetic constraints may function to retain the secondary structure of viral RNA. Also, amino acid sequences deduced from nucleotide sequences for coat proteins from CMV-As, -Y, -O and -Q were compared (data not shown). They showed that substitutions of amino acid residues to others basically lay in similar characteristics for example tyrosine residue in CMV-Y and -O strains to phenylalanine residue in CMV-As strain at position 99.

According to the nucleotide sequence comparisons, CMV-As is presumably included in WT-type CMV

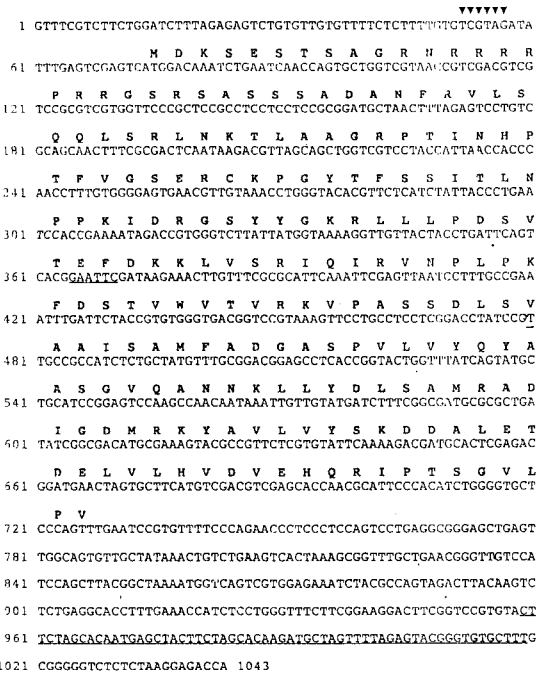

Fig. 3. Nucleotide sequence of cDNA from RNA4 of cucumber mosaic virus-As strain (CMV-As), the Korean isolate. The deduced amino acid sequence for coat protein was shown with bold-faced one-letter code over the nucleotide sequence. The hexanucleotide sequence, a possible 18S ribosome binding site (32), was marked with arrowheads. The *Eco*RI site as described in the text was underlined. The 3' end region of 61 nucleotides underlined showed an extreme divergence compared to those of other strains.

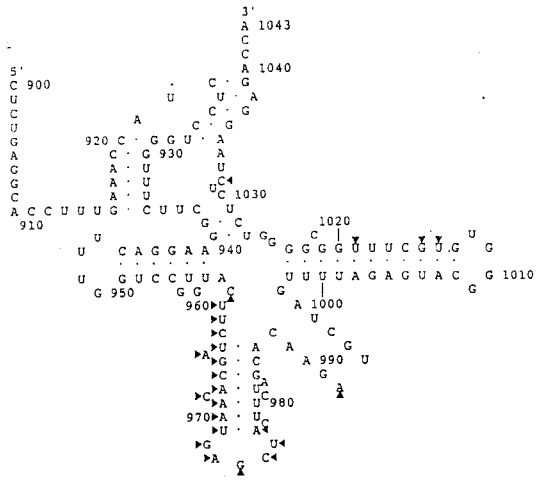

Fig. 4. The secondary tRNA-like pseudoknot structure formed at 3' end of CMV-AS RNA4. Structural dissimilarities on the primary sequence among the various isolates are indicated by arrowheads (4, 7, 16). The positions of bases were indicated by numbers.

Table 1. Comparison of nucleotide sequence of the CMV-As RNA4 with those of other strains

Viral strain	Degree of identity to CMV-As (%)				
	5' Leader region (1-73)[a]	3' Untranslated region (74-730)	Coding region (731-1943)	Overall	Type
CMV-Y	82.2	82.2	94.1 (94.0)[b]	89.1	WT
CMV-O	75.9	82.2	92.5 (94.0)	88.2	WT
CMV-M	94.0	81.6	93.6 (96.3)	89.9	WT
CMV-D	76.7	80.4	94.0 (97.2)	88.8	WT
CMV-I17F	83.1	90.0	94.4 (96.8)	91.9	WT
CMV-Fny	84.4	82.0	93.9 (94.5)	89.2	WT
CMV-Q	52.7	61.2	78.1 (72.0)	71.1	S

[a] Figures in parenthesis indicate the positions of nucleotides.

[b] Figures in parenthesis indicate the amino acid sequence identity in percentage.

such as CMV-Y, -O, -Fny, -M, -D, -C and -I17F, but different from S-type like CMV-Q and -WL.

tRNA-like structure of 3'-end noncoding region of CMV-As RNA4. It has been reported that RNAs 1 to 4 of CMV contain tRNA-like structure at their 3' non-coding regions (9). Although there are considerable variations in nucleotide sequences, the overall secondary structure of tRNA remains similar to those proposed for other strains of CMV (Fig. 4). They can be aminoacylated by tyrosine (11), and their functions were studied in detail for brome mosaic virus RNAs (2). When this region was deleted, the negative strand of RNA3 was not synthesized. Therefore, at the early stage of replication, the RdRp holoenzyme presumably recognizes the 3' tRNA-like secondary structure and binds to it for replication (6).

요 약

*Aster yomena*로부터 분리한 오이 모자이크 바이러스(cucumber mosaic virus) (CMV-As)의 RNA4로부터 완전한 길이의 cDNA를 합성하고 그 전체적인 염기서열(1,043 nt's)을 결정하였다. CMV-As RNA4는 73개의 염기로 구성된 5'말단의 leader 부위, 657개의 염기로 구성된 외피단백질(coat protein) 유전자 부위 및 312개의 염기로 구성된 3' 말단의 비번역 부위로 구성되어 있음을 확인하였다. 외피단백질 유전자 부위의 염기서열을 다른 계통의 CMV와 비교해 볼 때 그 염기서열이 보전적으로 존재하고 있으나 그 외의 부분은 다양함을 확인하였다. 특히 3' 말단부위의 61개의 염기로 구성된 부위(959-1019)는 다른 계통의 CMV에서는 상당히 유사하지만 CMV-As는 매우 달랐다. 하지만 3' 말단부위의 염기서열이 다른 계통과 많이 다름에도 불구하고 CMV-As도 다른 CMV처럼 tRNA와 유사한 구조를 역시 형성함을 확인하였다. CMV-As의 RNA4 염기서열을 다른 계통의 CMV와 비교할 때 CMV-I17F와 가장 유사하였으며(91.9%) S형의 CMV-M과는 가장 낮은 동일성을 보였다(71.1%). 이와 같은 염기서열의 비교 결과와 *Eco*RI 제한효소 인식부위의 존재로 미루어 CMV-As는 WT형으로 분류된다.

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