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Complete Nucleotide Sequences of RNA3s of Cucumber Mosaic Virus KM and D8 Strains

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Full-length cDNA clones of RNA3s of cucumber mosaic virus KM and D8 strain (CMV-KM and CMV-D8) were synthesized and their complete nucleotide sequences were determined. CMV-KM and CMV-D8 RNA3 consisted of 2210 and 2218 nucleotides, respectively, and contained predicted two open reading frames of 3a movement protein (MP) and coat protein (CP). Higher sequence similarities were observed at the nucleotide level between CMV-KM and CMV-Y (96%), CMV-KM and CMV-D8 (96%), and CMV-D8 and CMV-Y (97%), respectively, whereas lower similarities were found between CMV-KM and CMV-Q (76%), and CMV-D8 and CMV-Q(75%), respectively. Furthermore, there were almost complete sequence similarities (more than 97%) at the amino acid level among the MP (or CP) genes of CMV-KM, CMV-Y and CMV-D8. By contrast, lower amino acid sequence similarities were observed between the MP (or CP) genes of CMV-KM and CMV-Q (83%), and CMV-D8 and CMV-Q (82%), respectively. These results strongly suggested that both CMV-KM and CMV-D8 strains belong to Subgroup I in which CMV-Y is included.

INTRODUCTION

Cucumber mosaic virus (CMV), which is the type member of *Cucumovirus* in the family of *Bromoviridae*, is a positive-sense, single-stranded RNA plant virus. CMV has a tripartite genomic RNAs functionally divided into RNAs1, 2 and 3 in decreasing order of molecular weight (Mr). CMV RNA3 encodes 3a movement protein (MP) and coat protein (CP), but CP is translated from the subgenomic RNA4, which is generated from RNA3 during replication (reviewed in Paulkaitis *et al.*, 1992). Suzuki *et al.* (1991) experimentally demonstrated that MP played a crucial role for viral cell-to-cell movement and CP was indispensable not only for protection of viral genomic RNA but also for long-distance translocation in tobacco using biologically active *in vitro* synthetic RNAs transcribed from the cDNA clones of genomic RNAs of CMV-Y strain.

In molecular biological studies on the host-specificity of CMV, we have synthesized cDNA clones of genomic RNAs for a Cucurbita strain of CMV (CMV-KM) and a Brassica strain of CMV (CMV-D8). It was already reported that RNA3 of CMV-KM was involved in elicitation of severe chronic systemic mosaic in bottle gourd, and RNA2 and RNA3 of CMV-D8 were interdependently associated with introduction of systemic mosaic in radish (Takeshita *et al.*, 1995; Takeshita and Takanami, 1996). Differences in the nucleotide sequences and amino acid sequences encoded by the RNAs must direct the phenotypes of the CMV strains in the individual host plants. We therefore constructed the full-length cDNA clones of RNA3s of the both strains, determined their complete nucleotide sequences, and compared them with those of RNA3s of CMV-Y (Nitta *et al.*, 1988) and

CMV-Q (Gould and Symons, 1982).

MATERIALS AND METHODS

Virus and plants

CMV-D8 was originally isolated from a Japanese radish plant expressing systemic mosaic at a field in Kanagawa Prefecture in 1992, and CMV-KM was obtained from a melon plant showing mosaic symptoms in Kumamoto Prefecture in 1994, respectively. Virus was propagated in *Nicotiana tabacum* L. cv. Xanthi-nc and purified essentially according to Takanami (1981).

CMV RNA preparation and cDNA cloning

CMV RNA was extracted from the purified virus particles as described by Takanami (1981), and cDNA cloning was performed according to the standard or modified techniques (Sambrook *et al.*, 1989; Suzuki *et al.*, 1991; Boccard and Baulcombe, 1992). CMV RNAs were primed with an oligonucleotide 5'-TTT*GGCGGCCGC*TGGTCTCCTTTTGGAGG-3', which is complementary to the 3' end 17 nucleotides (underlined) of all CMV-Y RNAs (Kataoka *et al.*, 1990a, b; Nitta *et al.*, 1988) and contains a 3' nonviral sequences to creat a *Not* I site (italics). First-strand cDNA was synthesized with M-MLV reverse transcriptase and then subjected to a thermo-cycling amplification consisting of first step (2min 92 °C, 3min 42 °C, 3min 72 °C), second step with 30 cycles (1min 93 °C, 1min 40 °C, 3min 72 °C) and final step (3min 72 °C) using *Taq* DNA polymerase. The second primer used for amplification of the double-stranded cDNAs of CMV RNA3s was 5'-TTTT*GGATCC*AATTA-ATACGACTCACTATAGTAATCTAACCACCTGTGTGT-3', which contain a *Bam* HI restriction site (italics) and a T7 promoter sequence (double underlined) in the immediate upstream of the viral sequence (underlined) which is homologous to the 5' regions of CMV-Y RNA3. The DNA products were digested with *Bam* HI and *Not* I and inserted into the corresponding sites of pBluescript II KS+.

Sequencing of the cDNA clones of CMV-KM RNA3 and CMV-D8 RNA3

The nucleotide sequences of RNA3s of CMV-KM and CMV-D8 were determined by the dideoxynucleotide termination method essentially according to Sanger *et al.* (1977) using double-stranded plasmid DNAs as templates. Sequencing of the respective cDNA clones was begun with a thermo-cycling reaction using a commercially available sequencing kit containing the M13-Forward Fluorescein and the M13-Reverse Fluorescein primers (*Taq* Cycle Sequencing Kit for Shimadzu DNA Sequencer Ver. 2, Takara) and then analyzed in an automated sequencing facility (DNA Sequencer DSQ-1000, Shimadzu). The DNASIS (Hitachi) sequence analysis program was used in the analysis of CMV RNA3s. The complete nucleotide sequences can be found in the DDBJ data bases (Accession Nos. AB004780 for CMV-KM and AB004781 for CMV-D8).

RESULTS AND DISCUSSION

The complete nucleotide sequences of cDNA clones of CMV-KM and CMV-D8 RNA3s have been determined. The cDNA cloning and sequencing strategies for CMV-KM and

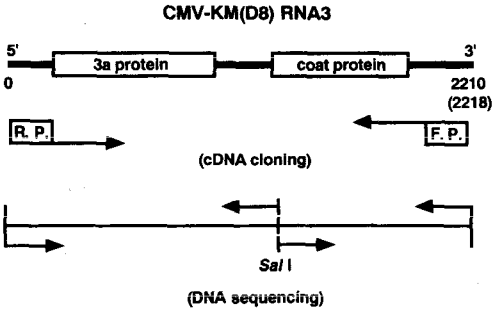

Fig. 1. Schematic representation of the strategy for cloning and sequencing of CMV-KM (D8) RNA3. Specific primers used for the synthesis of first strand cDNA (F. P.) and second strand cDNA (R. P.) are indicated by white boxes. The nucleotide sequences of individual primers are described in the Text. Arrows indicate the direction for the extention of the double-stranded cDNAs and sequencing.

CMV-D8 RNA3s and their number of nucleotides are shown in Fig. 1. and Table 1, respectively. Alignment of the complete nucleotide sequence of RNA3s of CMV-KM, D8, Y, and Q, is shown in Fig. 2 and the similarities among them in the sequences of nucleotide and amino acid of predicted proteins are summarized in Table 2. As shown in Table1, CMV-KM and CMV-D8 RNA3 contain 2210 and 2218 nucleotides (nt), respectively, with two open reading frames encoding the predicted MP (279 amino acids, Mr 30,474 and 30,447, respectively) and the CP (218 amino acids, Mr 24,103 and 24,032, respectively). RNA3s of CMV-KM and CMV-D8 consist of 120nt of the 5' non-coding region, 837nt of the MP genes, 299nt and 302nt of the inter-cistronic region, respectively, 654nt of the CP genes, and 300nt and 305nt of the 3' non-coding region, respectively (Table 1). Similarity in nucleotide sequences among RNA3s of CMV-KM, CMV-D8 and CMV-Y is 96~97%, whereas over all sequence similarities between RNA3 of CMV-KM and that of CMV-Q, and RNA3 of CMV-D8 and that of CMV-Q are 76% and 75%, respectively (Table 2). The amino acid sequence of the MP of CMV-KM RNA3 is identical to that of CMV-Y, while only one amino acid change was found in the deduced amino acid sequences of the MPs between CMV-D8 and CMV-KM, and CMV-D8 and CMV-Y. In contrast, the similarities in amino acid sequence of the MP ORFs between CMV-KM and CMV-Q, and CMV-D8 and CMV-Q are 83% and 82%, respectively. The CP gene encoded in CMV-KM RNA3 has 99% and 98% similarity at the amino acid level with those of CMV-Y and CMV-D8, respectively, and that of CMV-D8 share 97% similarity with that of CMV-Y. On the other hand, amino acid sequence identity of CP between CMV-KM and CMV-Q, and CMV-D8 and CMV-Q are only 83% and 82%, respectively. CMV strains have been reported to fall into Subgroup I and II on the basis of serology, nucleic acid hybridization and comparison of nucleotide sequence homology (reviewed in Paulkaitis *et al.*, 1992). In the same Subgroup, almost complete nucleotide sequence similarity has been shown to be conserved among CMV strains in genomic RNAs1, 2 and 3, respectively. Our results, therefore, clearly demonstrated that CMV-KM and CMV-D8 strains belong to subgroup I in

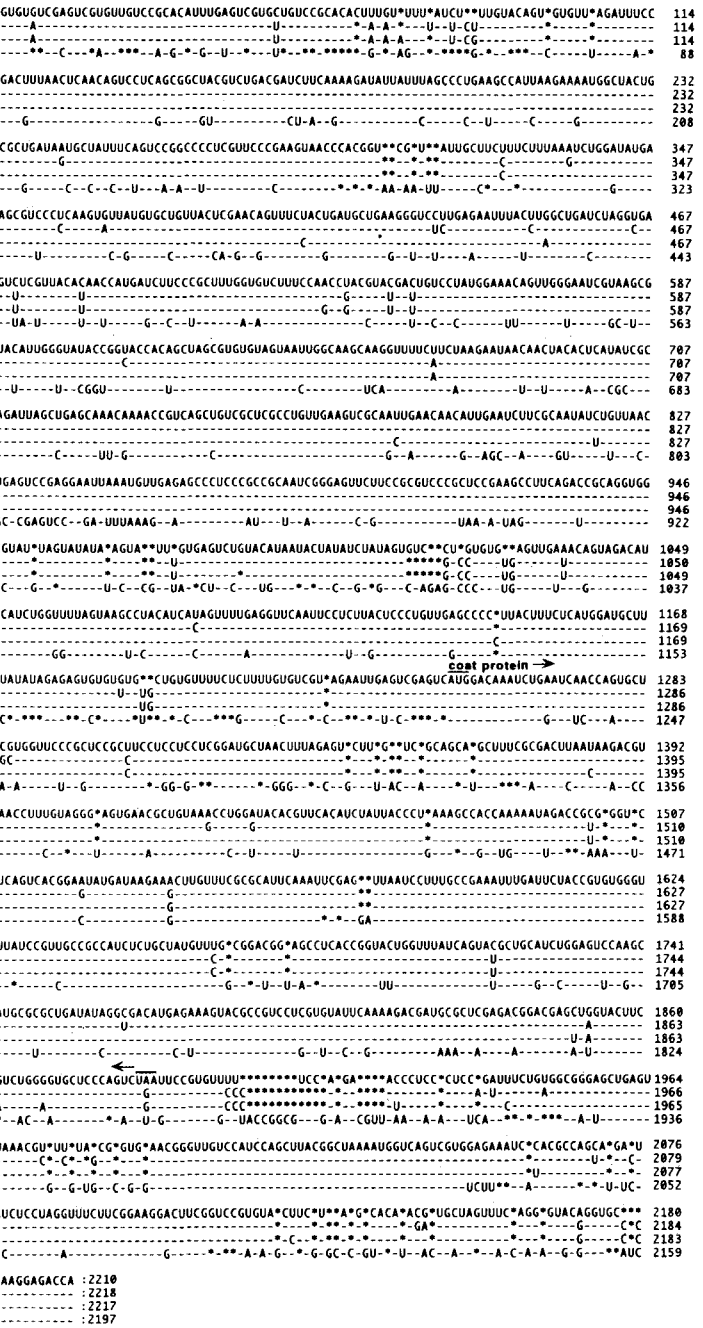

Fig. 2. Alignment of the nucleotide sequences of four CMV RNA3s; CMV-KM RNA3 (top line), CMV-D8 RNA3 (second line), CMV-Y RNA3 (third line) and CMV-Q RNA3 (bottom line). Nucleotides identical to CMV-KM RNA3 are indicated by (-). Deletions are indicated by (*). Initiation and termination codons for MP and CP are indicated by (—).

Table 1. Number of nuleotides in CMV-KM and CMV-D8 RNA3s

Virus strain	Region					
	5' Non-coding	3a protein	IR[a)]	Coat protein 3'	Non-coding	Total
CMV-KM RNA3	120	837 (279a.a.[b)])	299	654 (218a.a.)	300	2210
CMV-D8 RNA3	120	837 (279a.a.)	302	654 (218a.a.)	305	2218

a) Inter-cistronic region
b) Amino acid

Table 2. Nucleotide and amino acid sequence similarity among CMV RNA3s

CMV	KM vs. Y	KM vs. D8	D8 vs. Y	KM vs. Q	D8 vs. Q
Nucleotide sequence (%)	96	96	97	76	75
Amino acid sequence (%)					
3a protein (MP)	100	99	99	83	82
Coat protein (CP)	99	98	97	83	82

which CMV-Y is included, but not to Subgroup II to which CMV-Q belongs.

The predicted MPs of CMV-KM and CMV-Y have an Asn and that of CMV-D8 has a Ser at amino acid position 51. Furthermore, it was found that there are several amino acid differences in the sequence of the predicted CPs among the three CMV strains. Since these differences in the amino acid sequences among MPs and/or CPs must affect symptom induction, viral cell-to-cell and leaf-to-leaf spread in host plants, further molecular biological analysis using *in vitro* transcripts derived from the full-length cDNA clones and their recombinants will be indispensable for a precise understanding of the bases of the host-specificity of CMV.

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