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Identification and Sequence Analysis of RNA3 of a Resistance-Breaking *Cucumber mosaic virus* Isolate on *Capsicum annuum*

Mi Yeon Lee[1], Jang Ha Lee[2,3], Hong Il Ahn[1], Ju Yeon Yoon[1], Nam Han Her[2], Jang Kyung Choi[3], Gug Seon Choi[4], Do Sun Kim[4], Chee Hark Harn[2] and Ki Hyun Ryu[1]*

[1]*Plant Virus GenBank, Division of Environmental & Life Sciences, Seoul Women's University, Seoul 139-774, Korea*
[2]*Biotechnology Institute, Nong Woo Bio Co. Ltd., Yeoju 469-885, Korea*
[3]*Division of Biological Environment, Kangwon National University, Chunchon 200-701, Korea*
[4]*National Horticultural Research Institute, Rural Development Administration, Suwon 441-706, Korea*

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Cultivated hot pepper crops showing severe mosaic symptom were found in Korea in 2004 and their causal agent was identified as *Cucumber mosaic virus* (CMV). These pepper crops was resistant to the virus in the filed, and they belonged to pathotype 0 (P0) resistant pepper. Resistance screening of selected pepper plants showed that a pepper isolate of CMV was the P0 resistance-breaking virus. This P0 resistance-breaking isolate of CMV, named as Ca-P1, was isolated from leaves of the virus-infected *Capsicum annuum* cv. Manidda that showed systemic severe mosaic symptom. Ca-P1-CMV could induce systemic mosaic symptoms on P0-susceptible (P0-S) and P0-resistant (P0-R) cultivars whereas an ordinary strain (Fny-CMV) could not infect P0-R. This result suggests that Ca-P1-CMV can overcome P0 resistant pepper cultivars. To analyze its genome sequence, the complete nucleotide sequence of RNA3 of Ca-P1-CMV was determined from the infectious full-length cDNA clone of the virus. RNA3 of Ca-P1-CMV consisted of 2,219 nucleotides. Overall sequence homology of RNA3-encoded two viral proteins (movement protein and coat protein) revealed high similarity (75.2-97.2%) with the known CMV strains. By sequence analysis with known representative strains of CMV, Ca-P1-CMV belongs to a typical member of CMV subgroup IB. The resistance and resistance-breaking mechanisms of pepper and counterpart CMV, respectively, remain to be investigated, which will enrich the genetic resources and accelerate CMV-resistant pepper breeding programs.

Keywords **:** *Cucumber mosaic virus, Cucumovirus*, pepper, pathotype, resistance-breaking

Pepper (*Capsicum annuum*) is an economically important vegetable giving rise to a very large phenotypic diversity depending on the country where it is cultivated and its intended use, such as fresh or cooked vegetable, ornamental, spice, or extraction of either its natural colorant for food dyes or capsaicin for use in medicine. Peppers are produced on about 1,646,000 ha in 2004 in worldwide, and 60.7% of which are grown in Asia (FAO STAT, 2004; http://faostat.fao.org)

Over twenty-four viruses infecting peppers have been reported to cause economic problems in pepper production (Brunt et al., 1996). Among the pepper-infecting plant viruses, *Cucumber mosaic virus* (CMV) has been detrimental to pepper production in Korea as well as worldwide because of the widest host-range (infecting over 1,000 plants species), aphid-transmissible and the ability of synergistic effects with unrelated plant viruses (Palukaitis et al., 1992; Palukaitis and Garcia-Arenal, 2003). CMV, a type species of the genus *Cucumovirus* in the *Bromoviridae* family, is a tripartite, positive sense RNA virus consisted of three genomic RNAs, RNA 1, RNA 2 and RNA 3. To initiate an infection in host plant, the three genomic RNAs are required to invade a single cell. RNA 1 encodes 1a protein that contains domains of methyltransferase and helicase for virus replication. RNA2 encodes 2a protein, the viral polymerase (Hayes and Buck, 1990) and 2b protein that is essential for virus movement and infectivity in some hosts (Ding et al., 1995). Dicistronic RNA 3 encodes the 3a protein (or movement protein: MP) for local virus movement and the coat protein (CP) for encapsidation. CMV RNA3 is related with symptom expression in tobacco plant and pathogenicity in squash and corn plant (Gal-On et al., 1994; Ryu et al., 1998). A number of isolates of CMV can be divided into the three subgroups IA, IB and II (Palukaitis et al., 1992; Palukaitis and Garcia-Arenal, 2003).

To control CMV disease in pepper, various studies have been extensively demonstrated by using transgenic pepper plants expressing CP or expressing PR-proteins (Shin et al., 2002a; 2002b). However, it is difficult to obtain transgenic peppers conferring CMV-resistance, mostly due to poor regeneration of the plant. Although a few full-length cDNA

*Corresponding author.
Phone) +82-2-970-5618, FAX) +82-2-970-5610
E-mail) ryu@swu.ac.kr

clones of CMV have been constructed (Rizzo and Palukaitis, 1990; Boccard and Baulcombe, 1993; Ding et al., 1994; Roossinck et al., 1999; Salanki et al., 1994), the infectious clones of CMV isolated from pepper plant are not available.

Cultivated hot pepper crops showing severe mosaic symptom were found in Korea in 2004 and their causal agent was identified as CMV. These pepper crops was resistant to the virus in the filed, and belongs to pathotype 0 (P0) resistant peppers. Here, we characterized the biological properties and pathogenicity on pepper plants and sequence of RNA3 of a pepper isolate of CMV was determined and discussed.

Materials and Methods

Source of viruses and virus inoculation. An isolate of CMV, we named as *Capsicum annuum* pathotype 1 (Ca-P1-CMV), was originally isolated from diseased hot pepper cv. Manidda in Kyunggi province in 2004. The virus was isolated and propagated in *Nicotiana tabacum* cv. Xanthi-nc through mechanical inoculation after serial propagations in *Chenopodium quinoa*. Fny-CMV, As-CMV and LS-CMV, stock cultures in the Plant Virus GenBank (Seoul, Korea), were used as controls in this study (Choi et al., 1999; Ryu et al., 1998). The tobacco leaves collected 10 days after inoculation were used for virus purification, as previously described by Peden and Symons (1979). The cotyledons of 3 kinds of pepper cultivars (cv. Manidda, cv. Chammana, and cv. Leejo) and a P1 accession were inoculated by saps from infected tobacco plants. Inoculated plants were grown in controlled greenhouse conditions with the temperature at 26°C (daytime) or 20°C (night).

Restriction analysis of CP gene of the virus. To amplify and analyze CP gene of the virus and that of other 3 known strains (Fny, As and LS) of CMV, RT-PCR was done with CMV-specific primers (Choi et al., 1999) as described previously. RT-PCR product was digested with *Hin*dIII, *Sac*II and *Xho*I, and restriction fragments were analyzed on a 1.2% agarose gel (Sambrook et al., 1989).

Full-length cDNA cloning of RNA3 of the virus. To construct full-length cDNA of RNA3 of Ca-P1-CMV, the viral RNA was extracted from the purified virions by sodium dodecyl sulfate (SDS)-phenol extraction method (Choi et al., 1999; Choi et al., 2004; Gal-On et al., 1994). The purified viral RNAs were used as a template for RT-PCR. Briefly, RT-PCR was carried out at 42°C for 60 min with a reverse primer (5'-AATT**CTGCAG**TGGTCTCCT-TTTRGAGGCC-3') containing *Pst*I site (in bold) and each specific forward primer containing *Bam*HI site and T7 RNA promoter, as described by Choi et al. (2003). The purified cDNA was ligated into pUC18 digested with the same restriction enzymes to generate construct pCa-P1-CMV3.

Determination of sequence of RNA3 of the virus and phylogenetic analysis. To determine RNA3 sequence of the virus, the clone was digested by using *Pst*I-SalI, SalI-*Bam*HI and *Kpn*I-*Bam*HI. The digested DNA fragments were cloned into pSK(–) vector digested by each combinational restriction enzymes, respectively. Transformation of *E. coli* and gene manipulation were performed by standard protocols (Sambrook et al., 1989). Nucleotide sequences of these subclones were determined by dye-termination methods (Sanger et al., 1977) on both directions, and was analyzed by using the alignments of corresponding sequences from *Cucumovirus*-representative strains through the DNAMAN software package program (version 5.1, Lynnon Biosoft, Quebec, Canada). Multiple sequence alignments were generated using the DNAMAN package, and phylogenetic trees were constructed by the neighbor-joining algorithm, based on calculations from pairwise amino acid sequence distances for protein analyses derived from the multiple alignment format. The horizontal branch lengths are proportional to the genetic distance, and the numbers at each point indicate bootstrap values. The data set was subjected to 1,000 bootstrap replicates.

Results and Discussion

Isolation and pathogenicity of P0 resistance-breaking isolate of CMV from pepper plant. In this study, we originally isolated a new isolate of CMV from hot pepper plants and some properties of the virus were analyzed. Cultivated hot pepper crops showing severe mosaic symptom were found in Korea in 2004 and their causal agent was identified as CMV. The pepper isolate of CMV, Ca-P1-CMV, induced typical mosaic symptom on tobacco on its uninoculated systemic leaves indistinguishable from Fny-CMV. To determine the pathogenicity of the virus induced on selected pepper plants, 10 seedlings from each of the cultivars and a P1 accession as shown in Fig. 1, were inoculated by sap preparations. Ca-P1-CMV could induce systemic mosaic symptoms on P0-susceptible (P0-S) and P0-resistant (P0-R) cultivars whereas an ordinary strain (Fny-CMV) could not infect to P0-R (Fig. 1). This result suggests that Ca-P1-CMV can overcome P0 resistant pepper cultivars.

Ca-P1-CMV belongs to subgroup IB. To analyze the virus genome RNA and classify subgroup, the complete nucleotide sequence of RNA3 of Ca-P1-CMV was determined from the full-length cDNA clone. RNA3 of Ca-P1-

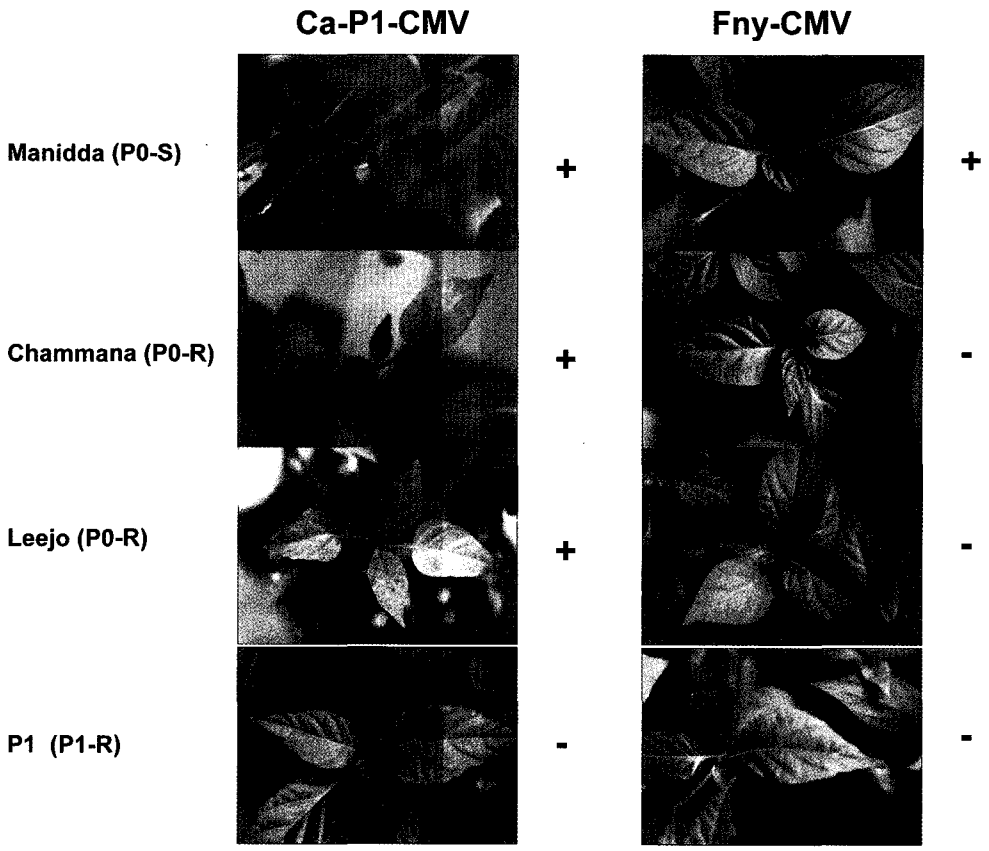

Fig. 1. Pathogenicity of Ca-P1-CMV as compared to Fny-CMV on 4 different *Capsicum annuum* plants. Infections of viruses were determined by RT-PCR and back inoculation methods. +: systemically infected; – : no infection.

CMV consisted of 2,219 nucleotides. The complete sequence of the virus has been deposited to the GenBank under DQ777747. Two viral proteins (movement protein (MP) and coat protein (CP)) encoded by the RNA3 revealed high similarity (75.2-97.2%) to those of other CMV strains (Table 1). By comparing the restriction patterns between Ca-P1-CMV and 3 selected strains of CMV, Ca-P1-CMV could be differentiated (Fig. 2). By sequence comparison with known representative strains of CMV based on phylogenetic tree analysis, Ca-P1-CMV belongs to a typical member of CMV subgroup IB (Fig. 3).

The RNA3 of Ca-P1-CMV, 2219 nucleotides in length is dicistronic and encodes 3a MP and CP separated by 297-nt long intercistronic region contained ICR-2-like conserved motif that is necessary for synthesis of subgenomic RNA like CMV and *Brome mosaic virus* (Boccard and Baulcombe, 1993, Smirnyagina, et al., 1994). The 3a MP of Ca-P1-CMV compared to the corresponding protein of CMV strains had approximate 82.7% to 97.5% amino acid sequence similarity (Table 1). The percentage amino acid sequence identity in the CP gene between Ca-P1-CMV and CMV strains ranged from 80.6% to 99.1%. The phylogenetic tree of CP indicates Ca-P1-CMV was closer to strains belonging to subgroup IB (Fig. 3). This result indicates that Ca-P1-CMV belongs to subgroup IB (Roossinck et al., 1999). In addition, 5'-noncoding region (NCR) of Ca-P1-CMV RNA3 contains the conserved UG-tract, like those of other cucumoviruses known to be *cis*-acting element for the accumulation of CMV RNA3 (Boccard and Baulcombe, 1993). The 3'NCR of the virus contains the highly conserved 40-nt sequence (5'-GAACGGGUGUC-CAUCCAGCUUACGGCUAAAAUGGUCAGU-3': from 2012 nt to 2051 nt in Pf RNA3) found in all cucumoviruses sequenced (McGarvey et al., 1995).

Although more experiments are necessary to determine viral proteins responsible for the pathogenicity of pepper and virus movement or host factors interacted with viral proteins, several studies with CMV and other viruses have implicated the polymerase protein as being involved in virus movement (Chen et al., 1996; Deom et al., 1997; Nelson and Van Bel, 1998). It is shown that two changes in the polymerase protein affected the elicitation of a hypersensitive response and restriction of CMV to the local lesion in cowpea (Kim and Palukaitis, 1997). In addition, both RNAs 2 and 3 is involved in host-specific infection in radish (Takeshita et al., 1998). There are many studies

Table 1. Sequence similarity (%) of nucleotide (nt) and amino acid (aa) between Ca-P1-CMV and other known strains of CMV[a]

CMV isolate	5'NCR	MP	MP	CP	CP	3'NCR
	nt	aa	nt	aa	nt	nt
207	89.4	94.6	93	96.3	92	85.3
CS	95.8	94.6	94.6	95.9	91.5	87.6
D8	84.9	94.6	93	95.9	91.9	86.3
Fny	88.5	94.6	93	96.3	92.2	86.2
I17F	87.2	93.9	92.5	95.9	91.7	84.9
KM	88.5	95	93	96.3	91.7	87.1
Kor	89.6	94.6	92.5	92.2	90	82
M	88.3	93.9	92.6	93.5	91.9	85.9
Mf	87.5	94.6	83.5	96.8	92.3	87.3
N	89.5	95.3	93.1	95.9	92.5	86
Ns	89.4	94.2	93.1	96.3	91.6	86
Ny	88.5	85.1	–	96.3	92.2	85.6
O	93.8	94.6	93	95.9	91	88.6
Pepo	87.2	95	93	94.9	91.1	89
Pf	80.6	94.2	92.4	95.4	91.6	86.1
Ri-8	82.5	94.6	93	96.3	91.9	84.6
Rs	89.4	94.6	93.1	96.3	92.3	86
Si	86	93.9	92	95.4	91.3	86
Twa	75.9	93.9	93	95.9	91.6	86
Y	85.1	95.3	93	93.5	91.9	84.6
AN-In	–	–	–	95.9	91.3	–
As	94.9	95.7	95.8	96.3	91.6	81.4
Black_PEP-IN	–	–	–	97.2	92.6	–
C7-2	76.5	93.5	90.9	96.3	91.3	85.7
CN03-CN	–	–	–	99.1	96.6	–
IX	84.7	93.9	91.8	95.9	92.2	90.7
KS44	–	–	–	96.8	92.4	–
M48	92.7	97.5	95.1	93.5	90.3	89.4
NT9	84.9	95.7	93.9	96.8	92.8	89.3
P1-1	84	95	93.5	96.3	92.5	87.9
P3613TW	–	–	–	96.3	95.4	
Phy	84.9	95.7	94.5	96.3	91.9	88.3
RT06-GR	–	–	–	95.9	92	–
RT144-BG	–	–	–	96.3	91.1	–
RT54-CH	–	–	–	96.3	91	–
TR15-TH	–	–	–	96.3	92.8	–
Kin	64.7	83.4	77.5	81.9	76.1	64.2
LS	64.2	82.7	77.2	81.5	75.8	64.1
M2	66.2	83.4	77.4	81.9	76	64.5
Q	64.7	82.7	76.6	82.4	76.2	68.5
R	58.2	83	77.2	81.5	75.8	64.5
TRK7	66.2	83.8	77.4	80.6	75.2	64.2
Xb	65.7	84.1	77.9	81.5	76.1	64.5
TAV	63.1	65.7	64.4	42.6	53.2	55.1

[a]The nucleotide and amino acid sequence of isolates of CMV and TAV was obtained from the GenBank/EMBL/DBJ databases.

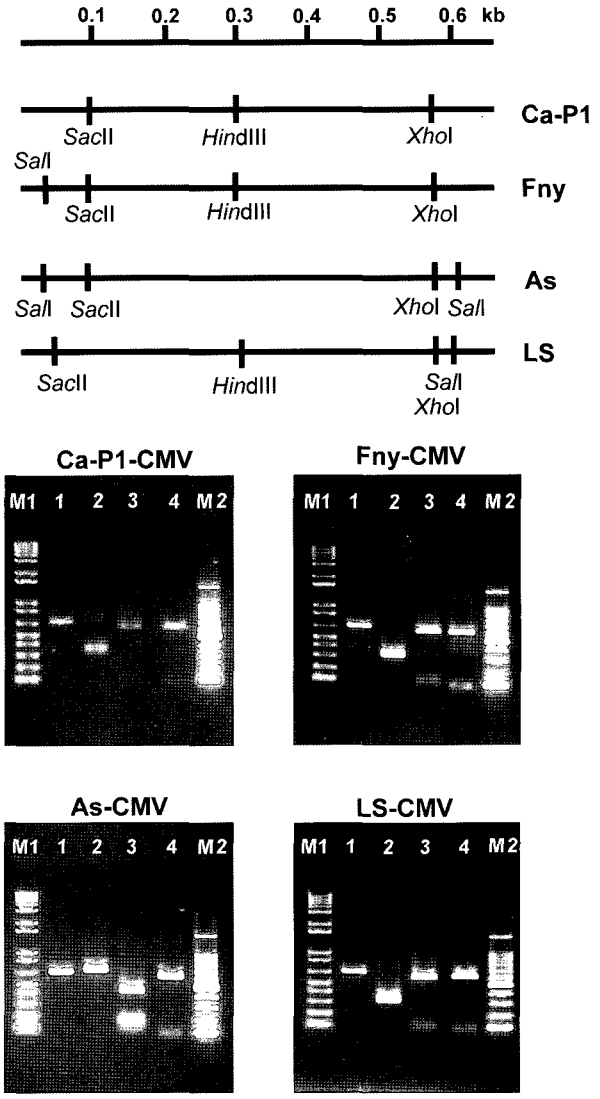

Fig. 2. Comparison of restriction pattern of RNA3 between Ca-P1-CMV and 3 other strains of CMVs by using RT-PCR amplicons of CP genes. Lane M1: 1 kb+ DNA ladder; 1: RT-PCR product (uncut); 2: *Hin*dIII; 3: *Sac*II; 4: *Xho*I; M2: 100bp DNA ladder.

showing that CMV MP and CP are involved in symptom production with complicated effect of cell-to-cell movement and systemic movement in host plants (Canto et al., 1997; Kaplan et al., 1997; Canto and Palukaitis, 1998). Although the involvement of CMV RNA3 on symptom expression and pathogenicity in other host plants (Gal-On et al., 1994; Ryu et al., 1998), there is no information on the interaction between CMV pathogenicity and pepper host resistance. The resistance and resistance-breaking mechanism of pepper and counterpart CMV, respectively, remain to be investigated, which will enrich the genetic resources and accelerate CMV-resistant pepper breeding programs.

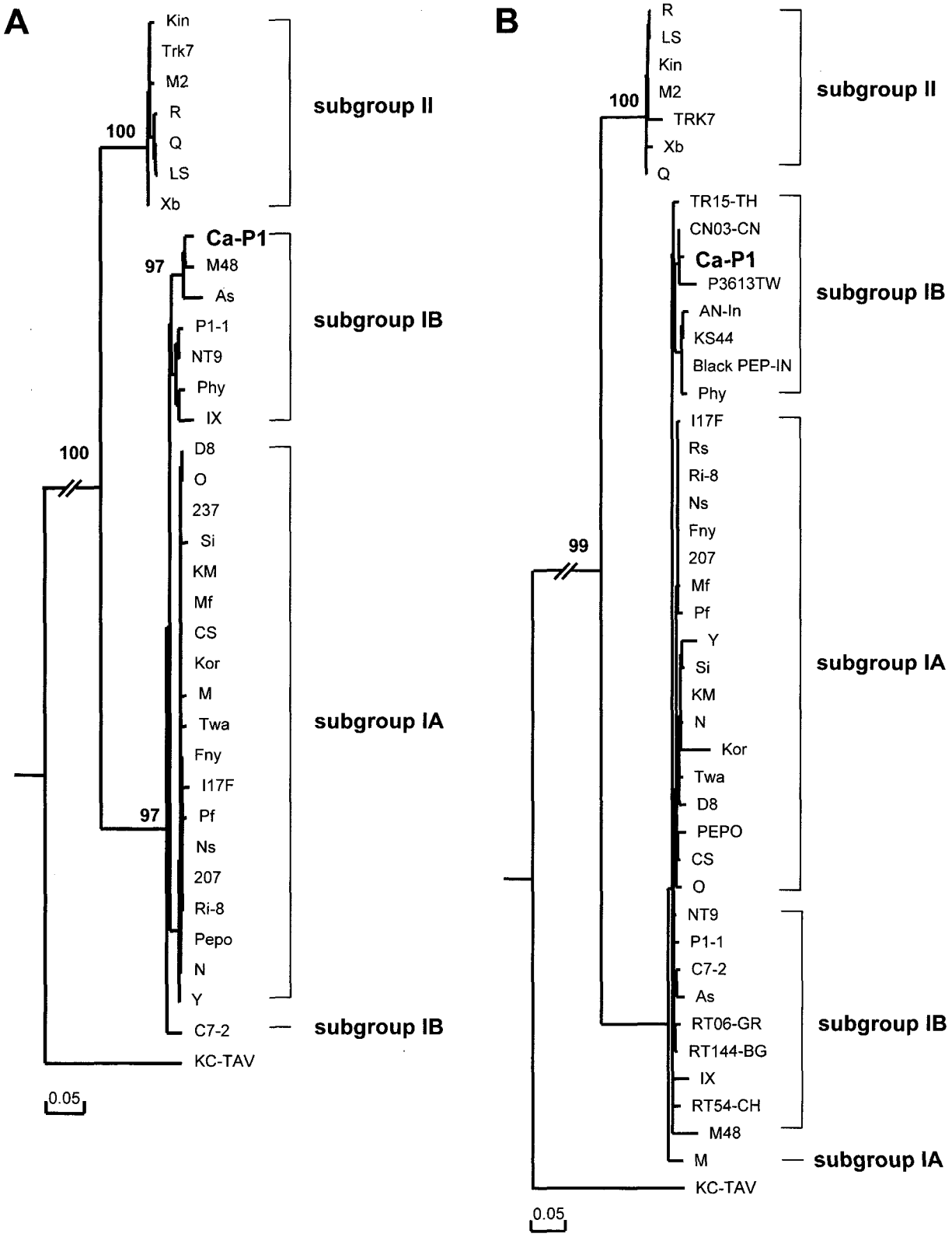

Fig. 3. Phylogentic tree analyses of Ca-P1-CMV and representative strains of CMV and a species of the genus *Cucumovirus* based on amino acid sequences of MP (**A**) and CP (**B**). The horizontal branch lengths are proportional to the genetic distance, and the numbers at each point indicate bootstrap values. The data set was subjected to 1,000 bootstrap replicates.

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