J. Plant Biol. 39(4) : 265~271 (1996)

Full-Length cDNA Cloning and Nucleotide Sequence Analysis of Cucumber Mosaic Virus (Strain Kor) RNA2

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Full-length cDNA for RNA2 of cucumber mosaic virus strain Kor (Kor-CMV) was cloned downstream of synthetic T7 promoter by reverse transcriptase-polymerase chain reaction (RT-PCR). The clone could generate a full-length transcript corresponding to RNA2 in size when synthesized by T7 RNA polymerase. The complete nucleotide sequence has shown that the RNA2 is composed of 3,049 nucleotides and contains one functional open reading frame (ORF) of 2,574 nucleotides encoding 2a protein. The deduced translation product of the 2,574 nucleotides contains GDD motif which is a characteristic of RNA-dependent RNA polymerase (RdRp). The amino acid sequence analysis of the 2a protein has shown that the homology is found in decreasing order with O-CMV (98.8%), Y-CMV (98.7%), Fny-CMV (98.3%), K-CMV (94.9%), Ix-CMV (91.9%), and Q-CMV (74.9%). Kor-CMV is suggested to belong to subgroup I in the aspect of nucleotide sequence homology of RNA2.

Keywords : CMV, cucumber mosaic virus, RNA2, 2a protein, RdRp

To protect economically important crops against plant RNA viruses, several pathogen-derived virus resistance strategies have been developed (reviewed by Wilson, 1993). The resistance strategies were to express virus-encoded replicase protein gene, coat protein gene or antisense RNA in transgenic plants. The strongest resistance was often shown in the plants expressing an intact or mutant form of the replicase protein (reviewed by Wilson, 1993; Anderson *et al.*, 1992), but the resistance was strain-specific as well as virus-specific (Zaitlin *et al.*, 1994). Therefore, it is necessary to clone and characterize the replicase gene of specific virus strain of interest.

Cucumber mosaic virus (CMV) infects a wide range of hosts exceeding 800 species including cucumber, cabbage, and pepper plants (reviewed by Palukaitis *et al.*, 1992). CMV is a single-stranded and plus-sense RNA virus of which genome is composed of RNAs 1, 2 and 3. RNAs 1 and 2 express 1a and 2a proteins, respectively, both of which are virus-encoded replicase components (reviewed by Palukaitis *et al.*, 1992). The purified viral RNA replicase from CMV-infected plants showed polymease activity upon addition of CMV RNA as template (Hayes and Buck, 1990). The 2a protein contains GDD motif that is highly conserved in RNA replicase (RNA-dependent RNA polymerase) which is essential in the life cycle of animal and plant RNA viruses (Kamer and Argos, 1984). The transgenic tobacco plants expressing a mutant form of the 2a protein could show effective resistance against CMV infection (Anderson *et al.*, 1992).

Therefore, the cloning of full-length cDNA for CMV will be very useful in the development of replicase-mediated resistance strategy and in the molecular study of RNA replicase *per se*. Additionally, it is also necessary that the cloned cDNA should be transcribed to generate an intact and functional viral RNA for the study of virus replication (Rizzo and Palukaistis, 1990).

In this study, RNA2 in Kor-CMV (Korean isolate) was cloned in the form of full-length cDNA that can generate an intact RNA2 by T7 RNA polymerase. Sequencing analysis of the cDNA revealed the complete nucleotide sequence of Kor-CMV RNA2, and

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phylogenetic relationships between Kor-CMV and other CMV strains are presented here.

MATERIALS AND METHODS

Virus strain and RNA extraction

Kor-CMV used in this study was supplied from Korea Ginseng and Tobacco Research Institute. The propagation and purification of Kor-CMV were described in the previous paper (Kim *et al.*, 1996). Kor-CMV RNAs were extracted with phenol buffered with TNE (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM Na_2-EDTA) from virus particles, and then precipitated by ethanol. The resulting RNAs were dissolved in diethylpryrocarbonate-treated water and quantitated by measuring absorbance at 259 nm.

cDNA synthesis

The cDNA of Kor-CMV RNA2 was synthesized using AMV reverse transcriptase (Poscochem.). The reverse transcription reaction was performed at 40°C for 3 h from 10 μg of purified RNA in a 50 μl reaction volume which contained 500 μM dNTP, 100 pmole of 3' MB primer [GACA(GGATCC : *Bam*HI) (ACGCGT : *Mlu*I)GGTCTCCTTTTGGAGG], RNasin (0.5 U/μl), AMV reverse transcriptase (1.0 U/μl), and reaction buffer supplied from manufacturer. Then, the reaction mixture was extracted with phenol/chlorform, and precipitated with ethanol. The resulting pellet was dissolved in 20 μl water which could be used at least 4 times for polymerase chain reaction.

Polymerase chain reaction and cloning

Five μl of the obtained cDNA mixture was amplified using *Dynazyme* (GENOMED). Polymerase chain reaction (PCR) was carried out in a 50 μl containing additional 100 pmole of 3' MB primer, 100 pmole of 1,2-5' primer [ACG(GGATCC : *Bam*HI) (TAATACGACTCACTATA : T7 promoter) GTTTTATTTACAAGAGCGT], 500 μM dNTP, 5 unit of *Dynazyme*, and reaction buffer supplied from manufacturer. PCR was performed in a thermal cycler (Perkin Elmer Cetus) for 20 cycles with each cycle consisting of 94°C for 1 min, 60°C for 0.5 min, and 72°C for 3.5 min. The amplified products were digested with *Bam*HI, and then electrophoresed on 0.8% agarose gel. The PCR product corresponding to approximately 3 kb was eluted with JETSORB kit (GENOMED), and then ligated into pUC18. The resulting plasmid was designated as pT7Kor2T.

In vitro transcription

The pT7Kor2T purified with Wizard Midipreps DNA purification system (Promega) was linearized with *Mlu*I digestion, and then transcribed by T7 RNA polymerase (Promega). *In vitro* transcription was performed at 37°C for 1 h from the linearized template in a 50 μl reaction volume that contained T 7 RNA polymerase (1.0 U/μl), 500 μM NTP, and RNasin (0.5 U/μl). The heat-denatured transcripts were electrophoresed on TAE-buffered agarose gel, and stained with ethidium bromide.

Sequencing analysis

The pT7Kor2T was digested with *Sal*I and *Sph*I, and serially deleted with Erase-a-Base system (Promega). The deleted cDNAs of various length were sequenced by dideoxy chain termination method using Sequenase Version 2.0 DNA Sequencing Kit (USB). For the sequencing of the other strand, the insert cDNA of the pT7Kor2T was subcloned in the opposite orientation by ligation after *Bam*HI digestion, resulting in pT7Kor2TR. The pT 7Kor2TR was digested with *Sph*I and *Mlu*I, serially deleted from the 3' terminus of RNA2 cDNA, and then sequenced.

Computer analysis

The PC/Gene (Intelligenetics) package programs were used in the analysis of CMV genomic RNAs and phylogeny. The published sequence sources of RNA2s used in computer analysis are following: strain Q (Rezaian *et al.*, 1984), Fny (Rizzo and Palukaitis, 1988), Y (Kataoka *et al.*, 1990), O (Hase *et al.*, 1992), K (Hellwald and Palukaitis, 1994), and Ix (McGarvey *et al.*, 1995).

RESULTS AND DISCUSSION

Strategy for the cloning of the full-length cDNA

Two primers used in the reverse transcriptase-polymerase chain reaction (RT-PCR) were specifically designed to clone the complete cDNA for RNAs 1 and 2 of Kor-CMV (Hays and Buck, 1993). The one is 1,2-5' primer that contains *Bam*HI site, T7 promoter sequence, and 5' terminal sequence. The 5'

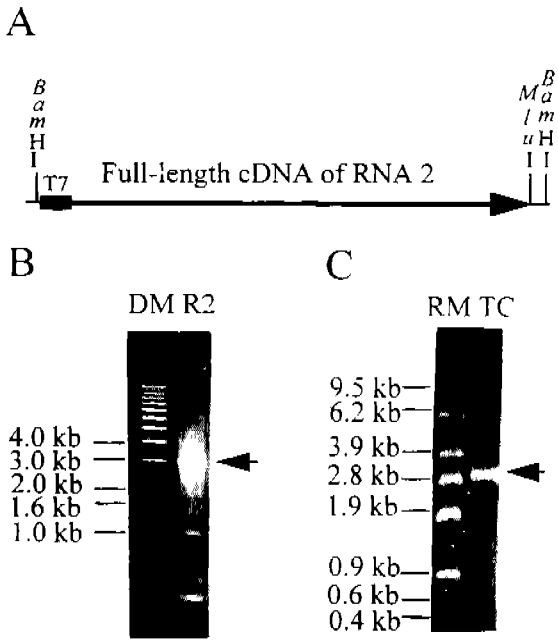

Fig. 1. Full-length cDNA cloning of Kor-CMV RNA2. (A) Schematic representaion of pT7Kor2T that contains the cloned full-length cDNA of Kor-CMV RNA2. T7 indicates synthetic T7 promoter. (B) Agarose gel (0.8%) electrophoresis of RT-PCR products amplified from the first strand cDNA synthesized from CMV RNAs. 1,2-5' primer and 3' MB primer were used in the PCR amplification. DM and R2 indicates 1 kb size marker and RT-PCR products, respectively. (C) Agarose gel electrophoresis of *in vitro* transcripts from the pT7Kor2T. *In vitro* transcription was performed by T7 RNA polymerase from the *Mlu*I-linearized pT7Kor2T, and the resulting transcripts were electrophoresed on 1.0% agarose gel. RM indicates RNA size marker; TC, *in vitro* transcripts.

terminal sequence is invariably conserved among various CMV strains and highly conserved between RNAs 1 and 2. The 1,2-5' primer was thought to favor the amplification of the cDNA of RNA1 rather than RNA2 because the primer would have perfect match with the 5' terminus of the RNA1 in comparison with one T insertion (5'-GTTTTA-) at 5'-GTTTA- of 5' terminus of the RNA2. Interestingly, the major PCR product was corresponding to RNA2 (3.0 kb) rather than RNA1 (3.4 kb) in our PCR condition (Fig. 1B). This reflects that the size difference rather than the slight Tm difference might be an important factor in the first-strand cDNA synthesis and PCR. The 5' half cDNA fragment of RNA1 could be amplified selectively with the use of 3' primer which is highly conserved minus-sense sequence in the middle of RNA1 (unpublished result). The T7 promoter sequence in the 1,2-5' primer was designed to initiate transcription at the G of the 5'-GTTTTA- which is thought to be the 5' proximal sequence of Kor-CMV RNA2 except viral cap structure (Fig. 2).

The other is 3' MB primer that contains *Bam*HI site, *Mlu*I site, and the complementary sequence of 3' terminus. The *Bam*HI sites in the 1,2-5' primer and 3' MB primer were inserted on purpose for the easy cloning of the amplified cDNA with *Bam*HI digestion. The complementary sequence to the 3' MB primer was derived from the published sequence of Kor-CMV RNA3 because the 3' proximal sequence was thought to be highly conserved among RNAs 1, 2, 3, and 4 in the same virus strain (Kim *et al.*, 1996). The *Mlu*I cleavage of the cloned cDNA of RNA2 will terminate *in vitro* transcription at A of -CCA-3' which is thought to be the 3' proximal sequence of Kor-CMV RNA2. Therefore, it is possible to generate only virus sequence by *in vitro* transcription with the *Mlu*I-linearized template (Fig. 1A). Fig. 1C shows that the full-length transcript corresponding to RNA2 (3.0 kb) was generated by *in vitro* transcription from the pT7Kor2T which has the complete cDNA of RNA2.

Analysis of 5' and 3' untranslated sequences (UTRs) and coding regions

Kor-CMV RNA2 has 5' untranslated region (UTR) of 86 nucleotides, one functional coding region of 2574 nucleotides, and 3' untranslated region (UTR) of 389 nucleotides (Fig. 2). The 5' UTR contains conserved sequences homologous with internal control regions (ICRs) motifs that exist in the promoters of tRNA genes (Fig. 2; Marsh *et al.*, 1989). The ICRs motifs are also found in brome mosaic virus (BMV) which is another member of tripartite plant RNA viruses (Marsh *et al.*, 1989). The mutational analysis around the ICRs 1 and 2 in BMV has shown that the ICRs 1 and 2 are essential with the sequence- and structure-specific manner in the amplification of plus-sense RNA from minus-sense RNA (Pogue and Hall, 1992). From the viewpoint of similarity between CMV and BMV, the proposed structure of the 5' terminus is likely to be important in the replication of CMV RNAs 1 and 2 (Fig. 3A).

The translation product of the functional coding region of Kor-CMV RNA2 is 2a protein of 96.7 kDa which is a virus-encoded component of RNA-dependent RNA polymerase (RdRp). The 2a protein of Kor-CMV RNA2 contains "polymerase site" which is characterized by GDD and TGxxxTxxxNT motifs in RNA-dependent polymerases (Fig. 2; Morch *et al.*,

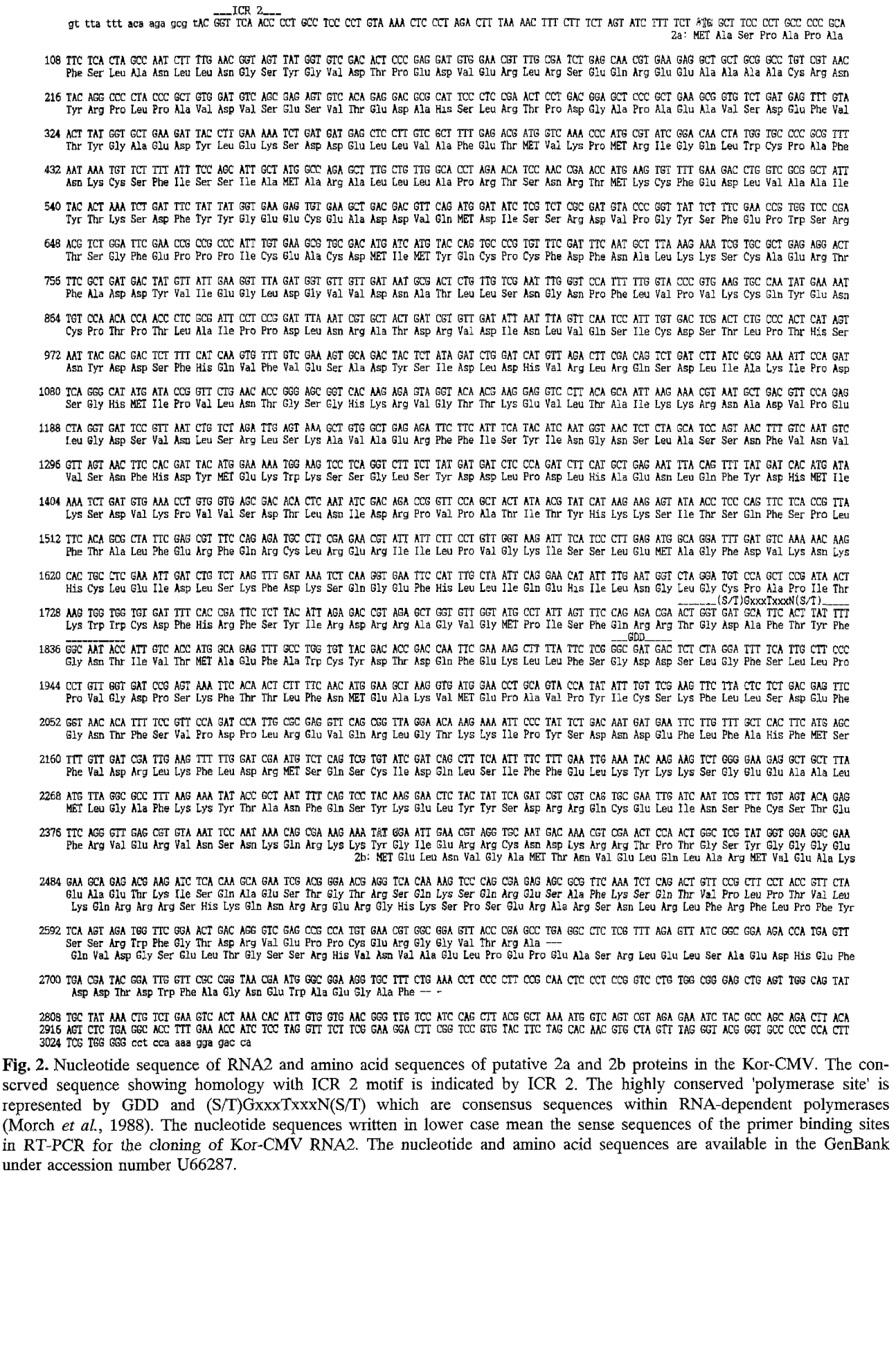

Fig. 2. Nucleotide sequence of RNA2 and amino acid sequences of putative 2a and 2b proteins in the Kor-CMV. The conserved sequence showing homology with ICR 2 motif is indicated by ICR 2. The highly conserved 'polymerase site' is represented by GDD and (S/T)GxxxTxxxN(S/T) which are consensus sequences within RNA-dependent polymerases (Morch *et al.*, 1988). The nucleotide sequences written in lower case mean the sense sequences of the primer binding sites in RT-PCR for the cloning of Kor-CMV RNA2. The nucleotide and amino acid sequences are available in the GenBank under accession number U66287.

1988). Additionally, Kor-CMV RNA2 contains homologous region with overlapping gene encoding 2b protein that has been found in Q-CMV (Fig. 2; Ding *et al.*, 1994). The 2b protein of unknown function is expressed only from a subgenomic RNA4A (Ding *et al.*, 1995). However, RNA4A was not found in tobacco leaves infected with Kor-CMV (unpublished result), so that the putative 2b protein of Kor-CMV is not likely to be expressed.

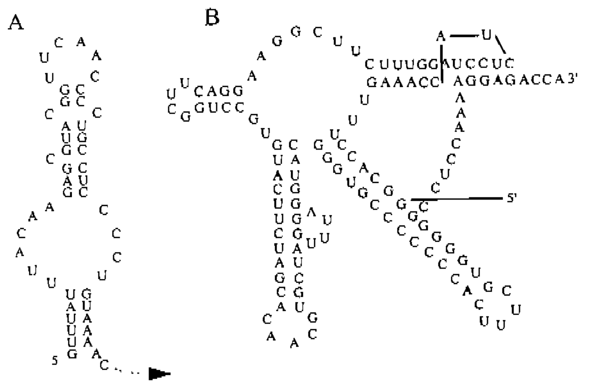

Fig. 3. Secondary structure models of plus-sense RNA sequences at the 5' (A) and 3' (B) termini of Kor-CMV RNA2. The structures were adapted from the similar models for Q-CMV RNAs by Joshi *et al.* (1983) and Pogue and Hall (1992).

The 3' UTRs of CMV RNAs 1, 2, and 3 contain the conserved sequences showing tRNA-like structure (Fig. 3B) that is thought to be the recognition site of viral RdRp. Although some base changes have been found in the 3' terminus of Kor-CMV RNA2 compared with those of Fny-, Y-, K-, Ix-, and O-CMV (data not shown), the whole structure of stem and loop is conserved well (Fig. 3B). Since the 3' terminal sequence of Q-CMV (subgroup II) is considerably different from that of Fny-CMV (subgroup I), it seems that the secondary structure is more important than the simple primary sequence in the function of 3' terminus (reviewed by Palukaitis *et al.*, 1992).

Analysis of nucleotide and amino acid sequence homology

CMV has been divided into two subgroups based on serological relationships, peptide mapping, and nucleic acid hybridization (reviewed by Palukaitis *et al.*, 1992). Kor-CMV RNA2 shows nucleotide sequence homology in decreasing order with O- (97.5%), Y- (97.3%), Fny- (97.2%), K- (92.0%), Ix- (90.0%), and Q-CMV (72.5%). From the nucleotide sequence homology, Kor-CMV is confirmed to belong to subgroup I (For RNA3, refer to Kim *et al.*, 1996)

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Q    MISPPPTFSFANLLNGSYGVDTPEEVERVRREQREDAEAALRNYKPLPAVDVSESVPRDEPIVSQTVTAAPVTSVDDAFVSFGAEDVLEMSPSELLSAFE  100
K    MAFPAPAFSLANLLNGSYGVDTPEEVERVRSEQREEAAAACRNYRPLPAVDVSESVPEDAHSL-RTPDGAPSEEVSVEFVTYGAEDYLEKSDDELLVAFE   99
Ix   MAFPAPAFSLANLLNGTYGVDTPEEVERVRSEQREEAAAACRNYKPLPAVDVGASVTEDAHSL-RTPDGAPSEEVSVEFVTYGAEDYLEKSDDELFVAFE   99
Fny  MAFPAPAFSLANLLNGSYGVDTPEDVERLRSEQREEAAAACRNYRPLPAVDVSESVTEDAHSL-RTPDGAPAEAVSDEFVTYGAEDYLEKSDDELLVAFE   99
O    MASPAPAFSLANLLNGSYGVDTPEDVERLRSEQREEAAAACRNYRPLPAVDVSESVTEDAHSL-RTPDGAPAEAVSDEFVTYGAEDYLEKSDDELLVAFE   99
Y    MAFPAPAFSLANLLNGSYGVDTPEDVERLRSEQREEAAAACRNYRPLPAVDVSESVTEDAHSL-RTPDGAPAEAVSDEFVTYGAEDYLEKSDDELLVAFE   99
Kor  MASPAPAFSLANLLNGSYGVDTPEDVERLRSEQREEAAAACRNYRPLPAVDVSESVTEDAHSL-RTPDGAPAEAVSDEFVTYGAEDYLEKSDDELLVAFE   99
     *  *.*.**.******.*******.***.*.****.*.** ***.*******..**. *.. . .* ..** ..*. .**..*******.* .**. ***

Q    LMVKPLRVGEVLCSSFDRSLFISSVAMARTLLLAPLTSTRTLKRFEDLVAAIYLKTDFFLED---DGPQTDVSQSDVPGYIFEPGQHSSGFEPPPICAK  196
K    TMVKPMRIGQLWCPAFNKCSFISSIAMARALLLAPRTSHRTMKCFEDLVAAIYTKSDFYYDDECEADDVQIDISSRDVPGYSFEPWSRTSGFEPPPICEA  199
Ix   TMVKPMCIGQLWCPAFNKCSFISSIAMARALLLVPNTPNRTMKCFEDLVAAIYTKSDFYYDDECEADDVQINISSRDVPGYSFEPWSRTSGFEPPPICEA  199
Fny  TMVKPMRIGQLWCPAFNKCSFISSIAMARALLLAPRTSHRTMKCFEDLVAAIYTKSDFYYSEECEADDAQIDISSRDVPGYSFEPWSRTSGFEPPPICEA  199
O    TMVKPMRIGQLWCPAFNKCSFISSIAMARALLLAPRTSHRTMKCFEDLVAAIYTKSDFYYSEECEADDAQIDISSRDVPGYSFEPWSRTSGFEPPPICEA  199
Y    TMVKPMRIGQLWCPAFNKCSFISSIAMARALLLAPRTSHRTMKCFEDLVAAIYTKSDFYYSEECEADDAQIDISSRDVPGYSFEPWSRTSGFEPPPICEA  199
Kor  TMVKPMRIGQLWCPAFNKCSFISSIAMARALLLAPRTSNRTMKCFEDLVAAIYTKSDFYYGEECEADDVQMDISSRDVPGYSFEPWSRTSGFEPPPICEA  199
      ****. .*.. *..*... ****.****.***.* *. **.* ********* *.**. ..     *. * ..* .***** ***  ..*********.

Q    CDLILYQCPCFDFNALRESCAEKTFSHDYVIEGLDGVIDNATLLSNLGPFLLPVHCSYSKTEDPDFVVDPSLARPTDRVDVHVVQAVCDTTLPTHGNYDD  296
K    CDMIMYQCPCFDFNALKKSCAERTFADDYVIEGLDGVVDNATLLSNLGPFLVPVKCQYEKCPTPTVANPPSLNRATDRVDINLVQSICDSTLPTHSNYDD  299
Ix   CNMIMYQCPCFDFNALKKSCAERTFADDYVIEGLDGVVDNATLLSNLGPFLVPVKCQYKQYPTPTVAIPPSLNRATDRVDINLVQSICDSTLPTHSNYDD  299
Fny  CDMIMYQCPCFDFNALKKSCAERTFADDYVIEGLDGVVDNATLLSNLGPFLVPVKCQYEKCPTPTIAIPPDLNRATDRVDINLVQSICDSTLPTHSNYDD  299
O    CDMIMYQCPCFDFNALKKSCAERTFADDYVIEGLDGVVDNATLLSNLGPFLVPVKCQYEKCPTPTIAIPPDLNRATDRVDINLVQSICDSTLPTHSNYDD  299
Y    CDMIMYQCPCFDFNALKKSCAERTFADDYVIEGLDGVVDNATLLSNLGPFLVPVKCQYEKCPTPTIAIPPDLNRATDRVDINLVQSICDSTLPTHSNYDD  299
Kor  CDMIMYQCPCFDFNALKKSCAERTFADDYVIEGLDGVVDNATLLSNLGPFLVPVKCQYENCPTPTLAIPPDLNRATDRVDINLVQSICDSTLPTHSNYDD  299
     *..*.**********..****.**..**********.************.**.* *..   .*.  .  *.*.*.*****...**..**.*****.****

Q    SFHQVFVDSADYSTDMDHVRLRQSDLVAKIPDGGHMLPVLNTGSGHQRVGTTKEVLTAIKKRNADVPELGDSVNLSRLSKAVAERFRLSYMNVDALAKSN  396
K    SFHQVFVESADYSIDLDHVRLRQSDLIAKIPDSGHMIPVLNTGSGHKRVGTTKEVLTAIKKRNADVPELGDSVNLSRLSKAVAERFFIPYINGNSLASSN  399
Ix   SFHQVFVESADYSIDLDHVRLRQSDLIAKIPDSGHMIPVLNTGSGHKRVGTTKEVLTAIKKRNADVPELGDSVNLPRLSKAVAERFFIAYINGNSLATSN  399
Fny  SFHQVFVESADYSIDLDHVRLRQSDLIAKIPDSGHMIPVLNTGSGHKRVGTTKEVLTAIKKRNADVPELGDSVNLSRLSKAVAERFFISYINGNSLASSN  399
O    SFHQVFVESADYSIDLDHVRLRQSDLIAKIPDSGHMIPVLNTGSGHKRVGTTKEVLTAIKKRNADVPELGDSVNLSRLSKAVAERFFISYINGNSLASSN  399
Y    SFHQVFVESADYSIDLDHVRLRQSDLIAKIPDSGHMIPVLNTGSGHKRVGTTKEVLTAIKKRNADVPELGDSVNLSRLSKAVAERFFISYINGNSLASSN  399
Kor  SFHQVFVESADYSIDLDHVRLRQSDLIAKIPDSGHMIPVLNTGSGHKRVGTTKEVLTAIKKRNADVPELGDSVNLSRLSKAVAERFFISYINGNSLASSN  399
     *******.*****.*.**********.*****.***.*********.***************************.**********  ..*.*  ..**.**
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Fig. 4. Alignment of the deduced amino acid sequences in the 2a proteins from the published nucleotide sequences of Q-, K-, Ix-, Fny-, O-, Y-, and Kor-CMV. Stars indicate the positions in the perfectly conserved amino acids. The positions in the chemically similar amino acids are indicated by '.'. Four conserved motifs in RNA-dependent polymerases are represented by A, B, C, and D (Poch *et al.*, 1987) and eight conserved motifs in RNA-dependent RNA polymseases are designated by I, II, III, IV, V, VI, VII, and VIII (Koonin, 1991). The nearly invariant amino acids in RNA-dependent polymerases are underlined (Poch *et al.*, 1987).

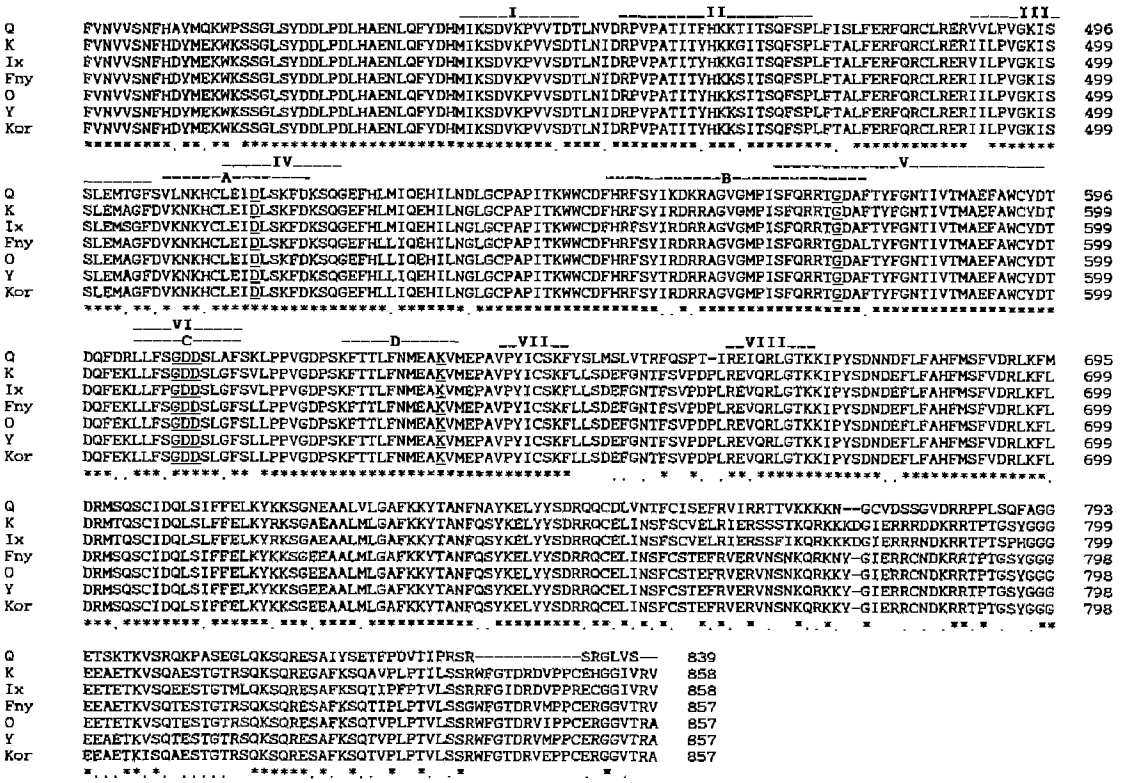

Fig. 4. Continued.

because Kor-CMV shows higher homology with Fny-CMV (subgroup I) than Q-CMV (subgroup II).

The amino acid sequence of the 2a protein of Kor-CMV was aligned with those of Q-, K-, Ix-, Fny-, Y-, and O-CMV (Fig. 4). The 2a protein (polymerase) is the only viral protein containing motifs conserved throughout plus-sense RNA viruses. Eight conserved motifs were identified from the linear alignment of RNA-dependent RNA polymerases from plus-sense RNA viruses (Fig. 4; Koonin, 1991), whereas only four motifs were found in RNA-dependent polymerases including RNA-dependent DNA polymerases (Fig. 4; Poch *et al.*, 1989). The central part that contains conserved motifs is more conserved than N-terminal or C-terminal portion (Fig. 4). The 2a protein of Kor-CMV shows the highest homology with that of O-CMV (98.8%) and the lowest homology with that of Q-CMV (74.9%). Additionally, the 2a protein of Kor-CMV shows more homology with those of Y-CMV (98.7%) and Fny-CMV (98.3%) than those of K-CMV (94.9%) and Ix-CMV (91.9%). The phylogenetic tree based on the amino acid similarity reflects that the 2a protein of Kor-CMV is very closely related to those of O-, Y- and Fny-CMV (Fig. 5). Additionally, the tree represents that the 2a protein of Q-CMV is distinctly different from the other CMV strains and the 2a proteins of Ix- and K-CMV are similar to each other.

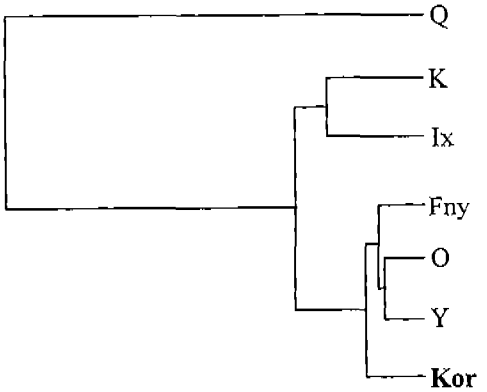

Fig. 5. Phylogenetic tree based on the amino acid similarities in the 2a proteins of Q-, K-, I-, Fny-, O-, Y-, and Kor-CMV RNA2.

ACKNOWLEDGMENTS

We thank Dr. Eun Kyung Park of Korea Ginseng and Tobacco Research Institute for the gift of Kor-CMV virions. This research was supported partly

from the Korea Research Foundation and from the Korea Ministry of Science and Technology.

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(Received December 5, 1996)