Nucleotide Sequence of the 3'-Terminal Region of Citrus Mosaic Virus RNA1

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ABSTRACT. The sequence of the 3'-terminal 2,615 nucleotides of citrus mosaic virus (CiMV) RNA1 was determined. The sequence contains a part of a single open reading frame (ORF) of 2,401 nucleotides and a 3' untranslated region of 211 nucleotides upstream of the poly(A) tail. The C-terminal region of the ORF is apparently homologous to the RNA-dependent RNA polymerase (RdRp) of viruses. The amino acid sequence of this region shows high similarity of 78% with RdRp of satsuma dwarf virus (SDV), suggesting a close evolutionary relationship between these viruses. Less sequence similarity was found with RdRps of parsnip yellow fleck virus (33%), rice tungro virus (31%), the genus *Comovirus* [cowpea mosaic virus (28%), cowpea severe mosaic virus (29%), red clover mottle virus (29%)] and the genus *Nepovirus* [grapevine chrome mosaic virus (28%), grapevine fanleaf virus (32%), tomato black ring virus (27%)]. There is no significant sequence similarity with other viruses, suggesting that CiMV and SDV are related to the viruses of the genera *Comovirus* and *Nepovirus*. However, the low similarity of RdRp suggests that CiMN and SDV are distinct from those viruses so far sequenced in these genera.

Index words. Citrus mosaic virus, satsuma dwarf virus, comovirus, nepovirus, RNA-dependent RNA polymerase.

Citrus mosaic virus (CiMV) is prevalent in some citrus-producing areas in Japan (33, 35). Infected satsuma mandarin develops green blotches on the rind of fruit at color break. CiMV has a wide host range in herbaceous plants (33). Field observations suggest that transmission occurs through soil, but no vector has been identified. The virus particles of CiMV are polyhedrons approximately 28 nm in diameter consisting of three centrifugal components in sucrose density gradients (33). The coat proteins consist of two components with M_r 42K and 23K (12). The genome of CiMV consists of two RNA species (RNA1 and RNA2), 7.0 kb and 5.4 kb, both of which are polyadenylated at the 3' termini (13). RNA2 encodes coat proteins, which show little amino acid sequence similarity with other sequenced viruses, at the 3' region (15).

A less characterized citrus mosaic with different symptoms and transmission mode was reported in India (1, 4, 21, 24). Indian citrus mosaic has been recently characterized as a badnavirus and given the name citrus yellow mosaic badnavirus (2).

CiMV is an unclassified virus, but the properties described above are similar to those of the comoviruses (3) and the nepoviruses (9). No serological tests have been conducted to compare CiMV, comoviruses, and nepoviruses. However, Usugi and Saito (31) reported that satsuma dwarf virus (SDV), which is similar to CiMV in biological (28, 29), morphological and serological (12, 33) properties, did not react to the antisera of comoviruses [bean pod mottle virus (BPMV), cowpea mosaic virus (CPMV)] and nepoviruses [arabis mosaic virus (ArMV), cherry leaf roll virus (CLRV), raspberry ringspot virus (RRV), strawberry latent ringspot virus (SLRSV), tobacco ringspot virus (TobRV), tomato black ring virus (TBRV), and tomato ringspot virus (TomRV)]. SDV is also unclassified (11, 17, 31, 32, 34) but tentatively classified to the genus *Nepovirus* (6). The SDV RNA-dependent RNA polymerase (RdRp) gene at the 3' region of RNA1 shows low but significant amino acid sequence similarity (25-28%) with those of the comoviruses and nepoviruses (14). In this report, we describe the cloning and sequencing of the 3' terminus of RNA1 of CiMV and compare the deduced amino acid sequence with those of the SDV, the como- and nepoviruses as well as other viruses.

MATERIALS AND METHODS

Viral RNA preparation. CiMV strain Ci-968K was propagated in *Physalis floridana* and purified as described previously with slight modifications (31, 33). Viral RNA was extracted from purified virions by the conventional SDS-phenol method and concentrated by ethanol precipitation. RNA was purified by oligo(dT)-latex treatment which is a modification of oligo(dT)-cellulose affinity chromatography (22).

cDNA synthesis and cloning. The first strand cDNA was synthesized using a mixture of RNA1 and RNA2 as templates and oligo(dT) as a primer, and the second strand cDNA was synthesized using RNase H, *Escherichia coli* DNA polymerase I and T4 DNA polymerase as described by Gubler and Hoffman (8). The double-stranded cDNA was ligated into the *Sma* I site of pUC19. The DNA was used to transform competent *E. coli* JM 109 cells, and the colonies were screened on LB medium with ampicillin, IPTG and X-gal.

Screening and analysis of cDNA clones. Selected white colonies were grown on a small scale, and plasmid DNAs were prepared by the simple single-step procedure (10). Recombinant plasmids with cDNA inserts greater than 1 kb were selected, eluted eletrophoretically from the agarose gel after digestion with *Pst* I and *EcoR I or Sac* I, and used as probes for Northern blot analysis with viral RNAs, using enhanced chemiluminescence (ECL) detection system (Amersham).

DNA sequencing. The recombinant clone CIK7 was selected for sequence analysis. The dideoxynucleotide chain termination reaction on the subclones of CIK7 was conducted with *Taq* DNA polymerase and -21M13 dye primer or M13 reverse dye primers (Applied Biosystems) using a DNA thermal cycler. The DNA sequence was analyzed in an automated DNA sequencer (373A, Applied Biosystems).

Computer analysis. Nucleotide and amino acid sequence data were analyzed and compared with those in the EMBL-GDB, GenBank, NBRF-PDB and SWISS-PROT, using GENETYX-MAC/CD software (Software Development Co., Japan).

RESULTS

Affinities to oligo(dT). Both CiMV RNA1 and RNA2 were successfully purified by oligo(dT)-latex treatment, suggesting that both RNAs contain poly(A) tails at the 3' termini.

Northern blot analysis. Restriction enzyme digestion showed that the insert of CIK7 has two sites for *EcoR* I and none for *Pst* I and *Sac* I. Northern blot analysis showed that the entire insert of CIK7 which was produced by digestion with *Pst* I and *Sac* I hybridized with both RNA1 and RNA2, whereas the *Pst* I and *EcoR* I fragment of CIK7 hybridized only with RNA1. Sequence analysis revealed that the fragment corresponds to the positions 1 to 1,914 in Fig. 1. These results show that CIK7 is complementary to RNA1 and that there is a region of strong sequence homology in the 3'-termini of RNA1 and RNA2. This 3'-conserved region of RNA1 and RNA2 was shown to be 203 nucleotides, of which 191 residues were found to be identical by sequencing the clone CIK7 and CIK11, the latter being specific to RNA2 (15).

Primary sequence analysis. The sequence of the 3'-terminal 2,645 nucleotides of RNA1 was obtained from subclones of CIK7. This region contains a part of one large open reading frame (ORF) end-

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        10        20        30        40        50        60        70        80
GATCCCTGCTATGAAATGTTGGGTAGTACCAAATAAAAACCCTGCGATTCCAGATGTAATGCGGAAAGTAACTTGCGAGA
 I  P  A  M  K  C  W  V  V  P  N  K  N  P  A  I  P  D  V  M  R  K  V  T  C  E  K

        90       100       110       120       130       140       150       160
AATCTAATCAGATCATTGATATATTTGCAAGCGAACTTTCGCGCTCTAGTGGTTGTGAGCCCGTTAGGCAATCACAAAGA
  S  N  Q  I  I  D  I  F  A  S  E  L  S  R  S  S  G  C  E  P  V  R  Q  S  Q  R

       170       180       190       200       210       220       230       240
TTTATTTATGCTGATGGGCCCGCTCGTAATGGTCATTGTGGTCGCCTTTTATGTGCTGAACTTTCCGGACACTGGCGCGT
F  I  Y  A  D  G  P  A  R  N  G  H  C  G  R  L  L  C  A  E  L  S  G  H  W  R  V

       250       260       270       280       290       300       310       320
TATAGGAATGTGTGCTGGTGAAGGCAAAGATTTAACAGGCACCACAAAAGCTCTTTACGCTGATATTCCAAGCGACTTTT
 I  G  M  C  A  G  E  G  K  D  L  T  G  T  T  K  A  L  Y  A  D  I  P  S  D  F  L

       330       340       350       360       370       380       390       400
TGGTTCCCGAGAACCCAAAAGCCGTTCATAGAGGAGCTGAAGTAGACGAGTCAATTCTCGATAGGTTCACAGTTTCCATT
  V  P  E  N  P  K  A  V  H  R/ G  A  E  V  D  E  S  I  L  D  R  F  T  V  S  I

       410       420       430       440       450       460       470       480
AAAATGGATGAACGAGTGTTGACTCCAATGACCAAAAGTTTAGGCAGGGTTTCTGGCCAGTTTCCCCGAGCCTTAAGAAA
K  M  D  E  R  V  L  T  P  M  T  K  S  L  G  R  V  S  G  Q  F  P  R  A  L  R  K

       490       500       510       520       530       540       550       560
GACGTCTATTGTCCCCTCCCTTATTAGCGAACATCTGTGGAGGAAGCCTGAAACTGAGCCGACAGTTCTGGGTAAGCGTG
 T  S  I  V  P  S  L  I  S  E  H  L  W  R  K  P  E  T  E  P  T  V  L  G  K  R  D

       570       580       590       600       610       620       630       640
ACTCTCGCACCCCTTACCCTTATGATCCTTACTCTTCAATCAGTGACAAATTTGTAGAGGAGGTTGGCCCCATCGATCTG
  S  R  T  P  Y  P  Y  D  P  Y  S  S  I  S  D  K  F  V  E  E  V  G  P  I  D  L

       650       660       670       680       690       700       710       720
AGCGCTGGCTCTCACGCCAATTTAGTTGTGGCCAATATAGGTTCATCGTGGAAATCAGCTGGCGAGATTCAGTGTGCACT
S  A  G  S  H  A  N  L  V  V  A  N  I  G  S  S  W  K  S  A  G  E  I  Q  C  A  L

       730       740       750       760       770       780       790       800
GGTTCTATCTTGGGACGTTGCAATTAATGGAGATCCAGCTATTCCATACTGTGAGAGACTCCCCATGTCCACCTCTGAGG
 V  L  S  W  D  V  A  I  N  G  D  P  A  I  P  Y  C  E  R  L  P  M  S  T  S  E  G

       810       820       830       840       850       860       870       880
GTTATCCCGACTCTATCTCACGGGCCTTTGGAGAAAAGGGGAAGAAGCGATTCTTCGATATGGATGGGAAAGTACTTAT
  Y  P  D  S  I  S  R  A  F  G  E  K  G  K  K  R  F  F  D  M  D  G  E  S  T  Y

       890       900       910       920       930       940       950       960
ATTCCCACCCCCGCTCTTTTGCAGGAGTTGGAGGTTCTTGAGAGGGAACTTCGGAAGGAAGAGGTGAGCTTAACTTGTAT
I  P  T  P  A  L  L  Q  E  L  E  V  L  E  R  E  L  R  K  E  E  V  S  L  T  C  I

       970       980       990      1000      1010      1020      1030      1040
AAATACAGCTTGCGCTAAAGACGAGAAAACCTCTTCAAAAAAGGTTAGGATTACACCTAAAACCCGCATATTTGAGATAC
 N  T  A  C  A  K  D  E  K  T  S  S  K  K  V  R  I  T  P  K  T  R  I  F  E  I  L

      1050      1060      1070      1080      1090      1100      1110      1120
TTCCTTTTCAAATCAATATCATTATAAGGAGGTATTTCATGTTCTGGATGCAGCTACTGATGGCTGCACATGATTTTTTG
  P  F  Q  I  N  I  I  I  R  R  Y  F  M  F  W  M  Q  L  L  M  A  A  H  D  F  L

      1130      1140      1150      1160      1170      1180      1190      1200
CCAGGTAAGGTGGGAATTAATGTTTATTCCGAAAGTTGGGATATCCTGCTTGGTAGACATACAAGGCTGGCTAATCACTT
P  G  K  V  G  I  N  V  Y  S  E  S  W  D  I  L  L  G  R  H  T  R  L  A  N  H  F

      1210      1220      1230      1240      1250      1260      1270      1280
TACTGGTGATTATTCAGGTTTTGACACATCCACTCCCAGAGTGTTGGTATACGCTATAGTTGATAAAATCAATGAATTAG
 T  G  D  Y  S  G  F  D  T  S  T  P  R  V  L  V  Y  A  I  V  D  K  I  N  E  L  A
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Fig. 1. Nucleotide sequence of the 3'-terminal 2,615 nucleotides of RNA1 of citrus mosaic virus (shown in DNA). The sequence is followed by a poly (A) tail. The predicted amino acid sequence of the single large ORF is presented below the nucleotide sequence. The restriction sites of *EcoR* I are shown by ▼ at the nucleotide positions 1,914 and 2,237. The four blocks of conserved motifs of RNA-dependent RNA polymerase (RdRp) are underlined (See text). The predicted cleavage site between RdRp and another protein is shown by a slash. The stop codon at the end of the large ORF is indicated by an asterisk.

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      1290      1300      1310      1320      1330      1340      1350      1360
CCAATGACTCTGAGGAGAATCAACGCACTCGTAGAAATCTCATCAGATTTGTTTTAAATCGCTATCTAATATCTGATGGA
  N  D  S  E  E  N  Q  R  T  R  R  N  L  I  R  F  V  L  N  R  Y  L  I  S  D  G

      1370      1380      1390      1400      1410      1420      1430      1440
TTTGTCTATGAAATACATGGTCGGGACACCTCTGGCTTTGCTCCTACTGTCATGATAAATTCAGTTGTCAATGAATTTTA
F  V  Y  E  I  H  G  R  D  T  S  G  F  A  P  T  V  M  I  N  S  V  V  N  E  F  Y

      1450      1460      1470      1480      1490      1500      1510      1520
TTTGAAATGGAGCTGGATGGGTCTAATGAAAGAGGCAGGGTATGCCCAACAGGCAACCCTTTATGCTTTTCATGAAGCCA
 L  K  W  S  W  M  G  L  M  K  E  A  G  Y  A  Q  Q  A  T  L  Y  A  F  H  E  A  T

      1530      1540      1550      1560      1570      1580      1590      1600
CTGAGATATCTCTATATGGTGATGATAACTTTGTCTCGGTGGCTACTCCTGTAGCCTCAATTTATAATCTGCAAACGATC
  E  I  S  L  Y  G  D  D  N  F  V  S  V  A  T  P  V  A  S  I  Y  N  L  Q  T  I

      1610      1620      1630      1640      1650      1660      1670      1680
TCTAATTTCCTATCAAGGGTTGGAGTTAAACTTGGAGATGGTGCCAAATCTGGTACTATCAAACCTTTTATACCCTTGGA
S  N  F  L  S  R  V  G  V  K  L  G  D  G  A  K  S  G  T  I  K  P  F  I  P  L  E

      1690      1700      1710      1720      1730      1740      1750      1760
AGAGGTTGATTTCCTGAAGCGGCAATTTGTAGCAGATTGTAGATCAACTGCTATTCTATGTCCATTAAAGAAAATCTCTA
 E  V  D  F  L  K  R  Q  F  V  A  D  C  R  S  T  A  I  L  C  P  L  K  K  I  S  I

      1770      1780      1790      1800      1810      1820      1830      1840
TTGAAGAGAGATTGTTTTATGTTAAGGGGGGTCAAGATGAATTAGCTGCTCTCGAGCTGAATATTGCGACGGCACTATGT
  E  E  R  L  F  Y  V  K  G  G  Q  D  E  L  A  A  L  E  L  N  I  A  T  A  L  C

      1850      1860      1870      1880      1890      1900      1910      1920
GAAGCCTTCTTCCATGGAGAGGAGTACTTCAAGGAATTAGAGTGCCGCATTAACAGAGCAGTTCATGGCAGTCGAATTCG
E  A  F  F  H  G  E  E  Y  F  K  E  L  E  C  R  I  N  R  A  V  H  G  S  R  I  R

      1930      1940      1950      1960      1970      1980      1990      2000
ACTGACTAAGCCCCTACCCACTATGGAGTCCATTAGGGTGTGGTACCTATCACAAAGATCGAATACGAAGGTACGCTCGC
 L  T  K  P  L  P  T  M  E  S  I  R  V  W  Y  L  S  Q  R  S  N  T  K  V  R  S  P

      2010      2020      2030      2040      2050      2060      2070      2080
CCTCCTACGAAGGCTTAGGAACGATGTCTGGAATACTCGCTGTGGGTATTTCAGAAGCTAAGAACGTAGGCGGAATCTCC
  S  Y  E  G  L  G  T  M  S  G  I  L  A  V  G  I  S  E  A  K  N  V  G  G  I  S

      2090      2100      2110      2120      2130      2140      2150      2160
TTCTTCTCCGGCACTGGTTTTCAAGGTAGAGTGGATAAACACCTTCAAGTGTTACCCACTTATATACCCGGAGGTTGGAG
F  F  S  G  T  G  F  Q  G  R  V  D  K  H  L  Q  V  L  P  T  Y  I  P  G  G  W  R

      2170      2180      2190      2200      2210      2220      2230      2240
AGTCAAGAAAGGACAAACCCAGATTAGTTTTATACGAGACGCCACCAAAATGGCTAGTATTTTTAAAATTCTGGGTGAAT
 V  K  K  G  Q  T  Q  I  S  F  I  R  D  A  T  K  M  A  S  I  F  K  I  L  G  E  F

      2250      2260      2270      2280      2290      2300      2310      2320
TCGAGTGTGTTGTAGCAACCGATAAATCGATGGCTTATATTGTTGCTATATGTTTGGCATATAAACATCGGAGGATTTCC
  E  C  V  V  A  T  D  K  S  M  A  Y  I  V  A  I  C  L  A  Y  K  H  R  R  I  S

      2330      2340      2350      2360      2370      2380      2390      2400
AGGATGGAAGTCAGATGTCATGTCCAGCTACTTAAAGTAGCAGATGCCCTGCTTTGCAACAGTATTTGTAACGGTCTCGA
R  M  E  V  R  C  H  V  Q  L  L  K  V  A  D  A  L  L  C  N  S  I  C  N  G  L  E

      2410      2420      2430      2440      2450      2460      2470      2480
GTAAGACCTCCTAGGTGATGCCCACCGGACCTTAACGTGGGTTTATATGTAAATACTAGTGTGTATAGCCTATTTGATTG
 *

      2490      2500      2510      2520      2530      2540      2550      2560
TTTTGTTATGTTTCTGAAAATAAACTGGATTTCAAAAAGGGTTTCTCAAACTCTTCCCTGTCCGATCCAGGAGTTTCGCG
      2570      2580      2590      2600      2610      2620
TGTTTTACTTGATTTCTGTCGCGTGCGGTTACGACCAGTGAAAGGCTGGTGGCCC(A)n
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ing with a UAA termination codon at nucleotide position 2,402 in one of the reading frames of the (+) strand (virion polarity), followed by a 3' untranslated region (3'UTR) of 211 nucleotides, and a 30-nucleotide-long tract of poly(A) tail. The nucleotide sequence and the deduced amino acid sequence of the protein encoded by this ORF are presented in Fig. 1. Other reading frames of the (+) and (-) strands contain many stop codons and few extended ORFs.

Properties of predicted protein. The putative amino acid sequence of the C-terminal region of the ORF was apparently homologous to RdRp of viruses (16). Four blocks of conserved motifs of RdRp (23) were found between nucleotide positions 1,187 and 1,654 (Fig. 1). Similarity was found with parsnip yellow fleck virus [PYFV (30)], rice tungro spherical virus [RTSV (27)], comoviruses [cowpea mosaic virus, CPMV (20), cowpea severe mosaic virus, CPSMV (5), red clover mottle virus, RCMV (26)] and nepoviruses [grapevine chrome mosaic virus, GCMV (18), grapevine fanleaf virus, GFLV, (25), tomato black ring virus, TBRV (7)]. Similarity was not detected with any other proteins in the databases.

The number of amino acid residues of the RdRp of CiMV was calculated to be 683, after the N-terminus of the RdRp was predicted by comparison with those of the como- and nepoviruses. The amino acid sequence of the predicted RdRp of CiMV showed 78% homology with SDV RdRp which consists of 682 amino acids, using the Homology program in the GENETYX software. Lower similarity values were obtained with those of PYFV (33%), RTSV (31%), comoviruses [CPMV (28%), CPSMV (29%), RCMV (29%)] and nepoviruses [GCMV (28%), GFLV (32%), TBRV (27%)]. These values were calculated in a conserved region of RdRp according to the algorithm developed by Lipman and Pearson (19), where the calculated region ranged from 420 (CiMV vs. GCMV) to 682 amino acids (CiMV vs. SDV).

The function of the N-terminal region of the part of the ORF could not be elucidated because the similarity with other proteins in the database was very low.

3' untranslated region. The 3' untranslated region (3'UTR) of CiMV RNA1 consists of 211 nucleotides, except for the poly (A) tail, and contains a putative polyadnylation signal AAUAAA at 117 nucleotides upstream from the poly (A) tail. This 3'UTR is longer than that of CPMV and shorter than those of the other como- and nepoviruses. Comparison of the 3'UTR of CiMV RNA1 did not reveal any significant sequence similarity with these viruses.

DISCUSSION

The strong similarity between the amino acid sequences of the putative RdRps of CiMV and SDV clearly shows an evolutionary relationship, which is in accordance with the similarity in biological and serological properties.

CiMV shares many common properties with the como- and nepoviruses, and this study and our previous one revealed that CiMV RNA1 and RNA2 encode RdRp and coat proteins in the 3' region, respectively, as do the como- and nepoviruses. Furthermore, RdRp of CiMV shows low but significant similarity (28-32%) with those of the como- and nepoviruses. However we cannot classify CiMV into the genus *Comovirus* or *Nepovirus* from this similarity because PYFV and RTSV, which are not como- or nepoviruses, shows similarities of 33% and 31%, respectively, with CiMV. In fact, the RdRp is highly conserved within the genera *Comovirus* and *Nepovirus*, except that homology between GFLV and TBRV is as low as 35%. However, the RdRp of TBRV is strongly similar (70%) to that of GCMV, a definite nepovirus. Low sequence similarity between GFLV and TBRV may suggest distinct sub-groups within the genus *Nepovirus*.

The presence of a poly (A) tail of RNA1 and RNA2 was confirmed in this study and the previous one (15), respectively, by sequencing. Northern blot analysis and sequencing revealed the presence of a region with strong sequence similarity in the 3' termini of RNA1 and RNA2. These features are similar to those of the como- and nepoviruses. However, the 3'UTR of CiMV has no similarity to those of the como- and nepoviruses.

In conclusion, we have identified a putative RdRp gene in the 3' region of CiMV RNA1. The high conservation of the amino acid sequence of RdRp between CiMV and SDV further confirms the close relationship which had been shown by biological and serological methods. CiMV and SDV share many properties including at least some gene organization which has been found for como- and nepoviruses. However, low RdRp sequence similarity between CiMV and the como- and nepoviruses, in spite of high conservation within the genus *Comovirus* or *Nepovirus*, suggests that CiMV and SDV should be classified into a new genus in the Family Comoviridae. Determination of complete sequences of RNA1 and RNA2 of CiMV and SDV is needed to confirm this hypothesis.

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