Complete nucleotide sequence of clover yellow mosaic virus RNA

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The entire genomic RNA of clover yellow mosaic virus was sequenced from cDNA clones and run-off cDNA transcripts. The genomic RNA is 7015 nucleotides in length [excluding a 3' poly(A) tail], with six open reading frames (ORFs) greater than 150 nucleotides in length. The first five ORFs encode proteins of Mr 191K, 26K, 12K, 6.5K and 28K, respectively. The sixth ORF lies completely within ORF1 and codes for a protein of Mr 14K. The capsid protein coding region (Mr, 23K) is found within ORF5 which encodes the Mr 28K protein. Proteins encoded by ORFs 1 to 3 and ORF5 show strong homology with proteins of other potexviruses, especially papaya mosaic virus.

Introduction

Clover yellow mosaic virus (CYMV) is a flexuous, filamentous plant virus of the potexvirus group. The genome consists of a linear positive sense ssRNA of Mr, 2.4 x 10^6 (Koenig, 1971) with a variable length poly(A) tail at the 3' terminus and a 5' m7GpppG cap structure (AbouHaidar, 1983). In vitro translation of genomic RNA produced a major polypeptide of Mr, 182000 (182K), as well as small quantities of 21K capsid protein (Bendena & Mackie, 1986). Similarly sized infection-specific polypeptides were detected in pea mesophyll protoplasts after inoculation with CYMV (Brown & Wood, 1987).

3' Coterminal subgenomic RNAs of 1-0 and 2-1 kb have been detected in CYMV-infected broad bean leaves (Bendena et al., 1987). Purified 1-0 kb subgenomic RNA produced CYMV capsid protein upon in vitro translation as well as a small quantity of 31K protein which was shown to be immunologically related to the capsid protein. A small proportion of the 1-0 kb capsid protein mRNA is encapsidated, as are those of the potexviruses narcissus mosaic virus (NMV) (Short & Davies, 1983; Mackie & Bancroft, 1986) and papaya mosaic virus (PMV) (Mackie et al., 1988). The coding properties of the 2-1 kb subgenomic RNA have yet to be elucidated. A third subgenomic RNA of 1-2 kb was also detected but was not extensively characterized (Bendena et al., 1987).

Recently, the sequence of the 3'-terminal 1050 nucleotides of CYMV RNA, which includes the coding region for the capsid protein, was reported (AbouHaidar & Lai, 1989). The complete nucleotide sequences of three other potexviruses have been reported: PMV (Sit et al., 1989), potato virus X (PVX) (Huisman et al., 1988) and white clover mosaic virus (WCIMV) (Forster et al., 1988). A virtually complete sequence of NMV RNA has also been reported (Zuidema et al., 1989). Each of these potexvirus RNAs contain five open reading frames (ORFs) with ORFs 2 to 4 overlapping adjacent ones. All of these genomes contain an intercistronic non-coding region between ORFs 4 and 5. With the exception of PMV, there is also a non-coding region between ORFs 1 and 2. In this paper, we report the complete nucleotide sequence of CYMV RNA and compare the genomic organization, nucleotide sequence and ORF products with those of other potexvirus, especially PMV, and carlavirus RNAs.

Methods

cDNA synthesis. CYMV was purified from broad bean plants (Vicia faba) as previously described (Bancroft et al., 1979) and viral genomic RNA (gRNA) was extracted as described by AbouHaidar (1988). First strand synthesis was primed with oligo(dT)12-18 or 5'-TATCACCC-CAAAAATCTACGGGT which is complementary to the first 23 nucleotides upstream from the poly(A) tail (AbouHaidar & Lai, 1989). Second strand synthesis was by the method of Gubler & Hoffman (1983). Double-stranded cDNA was either dC-tailed and annealed with PstI-cut, dG-tailed pUC18 (clone CH32) or methylated with EcoRI methylase and ligated into the EcoRI site of pSP64 (clones 15A, 56A and 40B).

DNA sequencing. Subclones were generated by restriction enzyme digestion. Double-stranded plasmid DNA was sequenced directly by the dideoxynucleotide termination method (Sanger et al., 1977) from
modified mini-preparations of plasmid DNA (Birnboim & Doly, 1979) grown in Escherichia coli strain HB101. Essentially, bacteria were lysed and plasmid DNA was precipitated with isopropanol. The DNA pellets were resuspended in 10 mM-Tris-HCl, 1 mM-EDTA pH 8.0 and treated with RNase A (10 μg) and RNase T1 (100 units) in a 100 μl reaction volume for 20 min at 37°C. Treated DNA was then passed through a Sephadex G50 spun column (Maniatis et al., 1982) and precipitated with 2 M-ammonium acetate and 2.5 volumes 95% ethanol at −20°C.

Purified plasmid DNA was linearized with a suitable restriction endonuclease prior to annealing with the sequencing primers. Sequencing reactions were performed with modified bacteriophage Φ17 DNA polymerase (Sequenase; U.S. Biochemical) (Tabor & Richardson, 1987) and [α-32P]dATP (Biggin et al., 1983) utilizing M13 universal and reverse primers (Pharmacia) as well as oligonucleotide primers specific for CYMV sequences. Linearized plasmid templates were boiled for 3 min with primer and cooled quickly on ice prior to the sequencing reaction. With the exception of the 5′-terminal sequences, sequences were determined using DNA in both orientations.

In order to determine the 5′-terminal residues, Maxam & Gilbert (1980) sequencing was carried out on terminal deoxynucleotidyl transferase (TdT) poly(dA)-tailed primer extension product. The procedure was as follows. A 32P-end-labelled synthetic primer 5′-GTTGGAGGGTGTTTACG, complementary to nucleotides 31 to 47, was annealed (in a 10-fold molar excess) to 5 ng of CYMV gRNA in buffer containing 175 mM-Tris-HCl pH 8.3, 175 mM-KCl, 35 mM-MgCl₂, by heating the mixture at 90°C for 3 min followed by incubations at 45°C for 30 min, 37°C for 15 min and room temperature for 10 min. The extension reaction was carried out in 10 mM-dithiothreitol, 250 μM each of dATP, dGTP and dTTP, 750 μM dCTP and 0.2 units/μl AMV reverse transcriptase and by adding 5 μg of CYMV gRNA. The extension reaction was carried out for 40 min, after heating of the reaction mixtures in 40% formamide, 8 mM-EDTA at 90°C for 3 min and electrophoresis in 10% polyacrylamide gel containing 8 M-urea, the band corresponding to the largest primer extension product was cut from the gel and eluted (Maxam & Gilbert, 1980). The eluted primer extension product, in 140 mM-potassium cacodylate pH 6.9, 30 mM-Tris base, 1 mM CoCl₂, 0.2 mM-2-mercaptoethanol and 4 mM-dATP, was incubated with 2 units/μl TdT at 37°C for 40 min, stopped with 10 mM-EDTA, phenol/chloroform-extracted and ethanol-precipitated twice. The poly(dA)-tailed primer extension product was then chemically sequenced following the instructions of the NEN Maxam and Gilbert sequencing kit.

RNA sequencing. A synthetic primer 5′-ATGCGAATCTTT-GCTGG, complementary to nucleotides 77 to 93, was annealed (in a fivefold molar excess) to 5 μg of CYMV gRNA or to 5 μg of chemically decapped CYMV gRNA (Flavell et al., 1982) under the conditions described above. The extension reaction was carried out in 9 mM-dithiothreitol, 0.4 units/μl AMV reverse transcriptase and 60 μCi [α-32P]dATP (sp. act. 1300 Ci/mmol). An aliquot of this mixture was immediately transferred to the termination mixtures which contained 100 μM (final concentration) each of dCTP, dGTP and dTTP, and one of the dideoxynucleotides, with a final concentration of 20 μM-ddCTP and -ddGTP, 40 μM-ddTTP, 3 μM-ddATP, or no ddNTP. The samples were incubated for 40 min at 45°C and then heated in 40% formamide, 8 mM-EDTA and analysed by electrophoresis.

Results and Discussion

Cloning and nucleotide sequence analysis

Two cDNA inserts (clones CH32 and 40B) spanned most of the CYMV genome, except for the 5′-terminal nucleotides (Fig. 1). Clones 15A and 56A overlap 40B and were used to confirm sequence data obtained from clone 40B. The extreme 5′-terminal nucleotides of CYMV RNA were sequenced directly by dideoxynucleotide sequencing with reverse transcriptase and by Maxam and Gilbert chemical sequencing of primer extension products. At the 5′ terminus of capped CYMV gRNA two G residues were detected by both methods. Prior chemical treatment of the RNA to remove the cap reduced the intensity of the more 5′ G by 70% (data not shown). We believe, therefore, that CYMV RNA contains a single 5′ G residue, as do other members of the potexvirus group (including PMV, PVX and WCIMV), and that the additional G was an artefact of reverse transcription (Ahlquist & Janda, 1984; Allison et al., 1988).

The complete nucleotide sequence of CYMV gRNA is shown in Fig. 2. The viral genome is 7015 nucleotides in length, excluding the poly(A) tail at the 3′ terminus which varies in length from 75 to 100 residues (Abou-Haidar, 1983). The gRNA of CYMV is the largest of the potexviral RNAs which have been sequenced.

Coding regions of the CYMV genome

The gRNA of CYMV contains six ORFs larger than 150 nucleotides (Fig. 1). ORF1 encodes a protein of Mr, 190651 (191K). The second coding region overlaps ORF1 by 20 nucleotides and produces a protein of Mr, 25800 (26K). However, mapping of the 5′ end of the 2.1 kb subgenomic RNA indicates that the second methionine is used as the start codon to produce a protein of Mr, 24850 (referred to as the 25K protein) (A. White & G. A. Mackie, unpublished results). ORF3 overlaps ORF2 by 35 nucleotides and ORF5 by one nucleotide, and encodes a protein of Mr, 12060 (12K). ORF4 overlaps ORF3 by 71 nucleotides and ORF5 by 107
Nucleotide sequence of CYMV RNA

1915

1916 MARVRAISLS

GAAAACAAAAACGAAAACAAACAAAACGAAAACAAACAAAAUCUUCGUAAACACCCUCCAACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCUCU

QFTDPSQKILIOQSDQYENVKKTGLATORYKMNYPYAHSESTAD

1921CCAACACCGGACCACCAUCAACAAAAACGAAAACAAACAAAAUCUUCGUAAACACCCUCCAACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

LEDLGIATNPFAAEEPTHTGAAKAIENDLYTIASRTMTKE

241CUACUAGGAAUGAAUGAGUAAGCAGAACCAACACCACACCACACCAGUUUCGCGCUUUGAAAAAACGAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

EPVTMFMMFKRAKLOYFRRGPPQNDTFINQIVPEPKDVAR

361GCAACACGUAACCUCAUGAUUGCUUGCUUCCACCCACCAUCCAUAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

EDTLHSTIPTIETKTVFIDOLFHLPPSFLTKLFARMPK

481GCAACACCCUCACCUCAACCUCACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

QTVLATMVLPTEALYGILTSLYPNWVSYLSYHKSFKRK

601CCCAAACGUAGAUGACGUAGUGGCUGGUCCACCCACCAUCCAUAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

FSYAPUGAEGAYELKQIDWLRFGHHIIGKVCTQAKL

721CUACUACUAUGACACGAGGAGCACAAAGGAGCGUUAAGAUGGUCGAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

ETKAANKHLFYRKYKFTEPFJRNFNTOTKIVVTLPKIFLPA

961GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

QFNHRVPIKKTLLVMLQFMYKVTVSAAKERDIWAKIRQL

121CCCAACGUAGAUGACGUAGUGGCUGGUCCACCCACCAUCCAUAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

TEDLQYESPTEIIVHLTVFLYFILAKTSAVTSMDLGSII

1241GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

KIKFRPWFYRGGYQRIFGGLFENQLLEALELSDELDLTL

1561GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

HPPAIAQKNITPSPEMSPSTOLLSSICALC5MSMDYAALEQR

1681GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

AABWKAEEEIQRGQKQQPLAVPPPPQTIKAQELAAAOIKOT

1801GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

EELIPSIPJHELTPALTEDLELDLEALQHITLEEVSSTSSV

2121GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

MRKTTEVEGLQOLQSSNCPTQRGLEEPTLTDLIKGR

2401GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

AAPHSRGGEPYSYTGFTHAQDPOWNOTLD1I1OQASFGOPTD

2561GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

FDHCLIOYNRHOYHRLPSDNEPCYPEAMPILTIINTEOQ

2281GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

EFIGSRRGEVTSYRLGPNSWLLMNPSGLQETHKXEVIMASE

2401GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

GRTSLFTFRSTKPLTQLPRTQKTEPTELPWKLLWGLV

2521GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

MNSSNFKNQGQRIGOLIPDIKDNKLPSLEYPHEL

2641GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

LEQLEKASRYCPYRIDIHKQGRAFASDVKNNRTGSTLTK

2761GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU

PDEWEKAEAMASPEQRTVLTVHAGGSGKSOLLORY

2881GAGAACCUAAAGGCAACCCCAUCAUCAACCAAACCUCCACACCAUUCAAUCGAAAUCAGAUCAGAAAUCGUUGCCGAGUCAGAUCUCUCU
Fig. 2. The complete nucleotide sequence of CYMV gRNA. The variable length poly(A) tail at the 3' end is indicated by An. The predicted amino acid sequences of the encoded proteins are shown above the respective nucleotide sequence. The sizes of the encoded proteins are indicated at the 5' ends of their respective ORFs.

nucleotides. ORF4 is +1 in relation to ORF3 and −1 in relation to ORF5 and encodes a protein of Mr 6470 (6.5K) whereas ORF5 encodes a protein of Mr 28093 (28K) (AbouHaidar & Lai, 1989). Unlike other potexviruses whose RNA has been sequenced, there is no intercistronic non-coding region between ORFs 4 and 5. There is also an additional ORF (ORF6) located completely within ORF1 in the +1 reading frame, which encodes a protein of Mr 13774 (14K).

Non-coding regions occur at the 5' and 3' termini. The 5'-terminal region is 94 nucleotides in length and the 3'-terminal non-coding region is 138 nucleotides in length, excluding the poly(A) tail.

The 5'-terminal 500 nucleotides of CYMV RNA were 49 to 54% identical to those of other potexviruses with PMV having the greatest degree of similarity. The nucleotide sequence at the initiation codon of the ORF1 proteins was invariably AUGGC, which resembles the consensus proposed by Lutcke et al. (1987) for plant mRNAs. However, nucleotides upstream of the AUG initiation codon differed between viruses.

Fig. 3 shows a comparison of the nucleotide sequences found upstream of the AUG initiation codons of the CYMV 25K, the 23K capsid protein gene and the 28K ORF. All potexviruses examined to date, including CYMV (Bendena et al., 1987), PMV and foxtail mosaic virus (FoMV) (Mackie et al., 1988), NMV (Short & Davies, 1983; Mackie & Bancroft, 1986), PVX (Dolja et al., 1987), WCIMV (Forster et al., 1987) and daphne virus X (Guilford & Forster, 1986) express their capsid proteins by means of a subgenomic RNA. The nucleotide sequence 5'-ACGGUUAAGUUUCCAU is located upstream of the PVX capsid protein ORF and a similar sequence is found to the 5' side of the PVX 25K ORF. This sequence may, therefore, be involved in the formation of subgenomic RNAs. The position of this sequence relative to the CYMV 25K initiation codon is consistent with the size of the 2.1 kb subgenomic RNA,
ACGCUUAA   ... (21nt)   ... AUG  PVX CP
ACGCUUAA   ... (25nt)   ... AUG  CYMV CP
uuGCUUAAc  ... (149nt)  ... AUG  CYMV 28K
ACcGUUAA   ... (23nt)   ... AUG  PVX 25K
ACcGUUAg   ... (47nt)   ... AUG  CYMV 25K.
ACGCUUcg   ... (52nt)   ... AUG  PMV 26K

Fig. 3. Comparison of nucleotide sequences upstream of AUG start codons of CYMV, PVX and PMV ORFs. The Y-terminal sequence of the PVX subgenomic RNA for the capsid protein (CP) (Dolja et al., 1987) is compared to similar sequences found upstream of various CYMV ORFs as well as the PVX 25K and PMV 26K ORFs. The position of these sequences to their respective initiation codons is indicated. Identical nucleotides are in upper case and bold typeface.

which can be detected in polyribosomal RNAs from CYMV-infected broad bean leaves (Bendena et al., 1987).

Similarity of CYMV proteins to other viral proteins

The FASTP computer program (Lipman & Pearson, 1985) was used to determine amino acid similarities among various potexviral polypeptides. The 191K product of ORF1 contains two regions having strong similarity to the ORF1 products of other sequenced potexviruses. The first region is about 400 amino acid residues in length and located towards the N terminus, whereas the second region of 800 amino acid residues is at the C terminus. The CYMV ORF1 product is the largest among those of the sequenced potexviruses: ORF1 in NMV is 186K, in PMV 176K, in PVX 166K and in WC1MV 147K. The difference in size of the various ORF1 products has been attributed to a central region of variable length between the two regions with a high degree of similarity (Zuidema et al., 1989). The percentage of identical amino acid residues ranges from 40 to 52% at the N termini and 50 to 54% at the C termini. The greatest degree of similarity was found between the two domains in CYMV and PMV. ORF1 also contains putative consensus sequences for NTPase-helicases (Skryabin et al., 1988) as well as for RNA polymerases (Argos, 1988).

The ORF2 (25K/26K) products produced by NMV, PVX and WC1MV are between 28 and 38% identical to that of CYMV but the 26K product of PMV is 47% identical to the CYMV 25K. Most similarity is centred around the region shown in Fig. 4(a). As with the other potexviral 25K/26K products, the CYMV 25K protein displays several conserved motifs of the NTPase-helicase consensus sequence (Hodgman, 1988; Skryabin et al., 1988).

The partial 3' sequence of the carlaviruses potato virus S (PVS) (MacKenzie et al., 1989) and potato virus M(PVM) (Rupasov et al., 1989), revealed the presence of three overlapping reading frames similar to the potexviral ORFs 2 to 4. When the CYMV 25K protein was compared to the PVS and PVM 25K proteins, 30 to 32% of the amino acids were identical, with the region of greatest similarity shown in Fig. 4(a).

The CYMV 12K protein is 34% identical to that of the NMV 14K product and 48% identical to that of the PMV 12K protein. Most of the similarity resides in the region seen in Fig. 4(b). The PVS 12K protein exhibits nearly as much similarity (47%) to the CYMV 12K protein as does the PMV 12K protein.

The ORF4 products are less similar, with an average of approximately 26% identity to the 6-5K product of CYMV. Once again, PMV is the exception with a degree of similarity of 36% and, surprisingly, the comparable PVS protein has about 35% identity, whereas PVM 7K protein only has 27% identical amino acid residues.

The conservation of sequence found in ORFs 2 to 4 of the potexviruses and carlaviruses further supports the inference made by Huisman et al. (1988) that one or more of the 25K, 12K or 8K proteins of PVX may be responsible for cell-to-cell viral transport.

A region of similarity was noted for the ORF6 products of CYMV and PMV, each encoded completely within their respective first ORF. The degree of similarity was about 39%, with most of the identical amino acid residues located within the sequence shown in Fig. 4(c).

Comparison of potexviral capsid protein sequences has been discussed previously (AbouHaidar & Lai, 1989). A striking similarity between CYMV and PMV is that the ORF encoding coat protein is found within an ORF that encodes a protein with additional amino acid residues at the N termini of the known capsid protein sequences. The CYMV ORF5 encodes a 28K polypeptide which has the capsid protein of 23K located within it. Should this be translated, there would be an additional 45 amino acid residues at the N-terminal of the coat protein including a second methionine residue located eight residues from the 28K start codon. This may correspond to the cross-reactive protein (31K on SDS gels) observed by Bendena et al. (1987). The PMV ORF5 encodes a 23K protein (Sit et al., 1989) with apparently five extra amino acid residues at the N terminus when compared to the amino acid sequence obtained directly from purified PMV capsid protein (Short et al., 1986). There is, unlike the case of CYMV, no in vitro evidence for the translation of these five extra amino acid residues. In vitro translation of FoMV gRNA produces a comparable 37K to 38K readthrough protein which is immunoprecipitable with coat protein antibody (Mackie et al., 1989).
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References


1988). In vitro translation of gRNA of PVS (MacKenzie et al., 1989) produces a 42K protein which resembles the CYMV 28K and PMV 23K proteins in that it has about 80 extra amino acid residues at the N terminus of the 33K capsid protein.

Fig. 4. Homology among CYMV-encoded proteins and other potexvirus and carlaviruses proteins. (a) Alignment of the CYMV 25K protein with other potexviral ORF2 products. The 25K proteins of the carlaviruses PVS and PVM are also shown for comparison. Gaps were introduced for best alignment. (b) Alignment of the CYMV 12K protein with other potexviral ORF3 products. The PVS and PVM 14K proteins are also included in the alignment. (c) Comparison of the 14K ORF6 products of CYMV and PMV. Both proteins are found in the +1 reading frame with respect to ORF1. Positions of amino acid residues within the respective polypeptides are indicated. Identical amino acid residues are in upper case and bold typeface.

Nucleotide sequence of CYMV RNA


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