

Nucleotide Sequence of RNA from the Sobemovirus Found in Infected Cocksfoot Shows a Luteovirus-Like Arrangement of the Putative Replicase and Protease Genes

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ABSTRACT

Ryabov, E. V., Krutov, A. A., Novikov, V. K., Zheleznikova, O. V., Morozov, S. Yu., and Zavriev, S. K. 1996. Nucleotide sequence of RNA from the sobemovirus found in infected cocksfoot shows a luteovirus-like arrangement of the putative replicase and protease genes. *Phytopathology* 86:391-397.

A virus found in naturally infected cocksfoot plants had a host range, symptom expression characteristics, and a particle morphology similar to those of cocksfoot mottle sobemovirus (CoMV). The viral RNA was cloned and sequenced (GeneBank accession number L40905). Computer-assisted analysis of the 4,083-nt RNA sequence revealed four open reading frames (ORFs) flanked by 5'- and 3'-untranslated regions of 69 and 228 nt, respectively. The 3'-proximal ORF putatively coded for a 27.6-kDa

product that was obviously homologous to the coat proteins of southern bean mosaic sobemovirus (SBMV) and rice yellow mottle sobemovirus (RYMV), which agrees with the identification of this virus as a Russian isolate of CoMV. This virus genome is closely related to that of a recently sequenced Norwegian isolate of CoMV. The 5' end of the subgenomic RNA was mapped 35 nt upstream of the coat-protein ORF initiation codon. Despite the considerable sequence similarity of the CoMV coat protein, putative RNA-dependent RNA polymerase (RDRP), and protease with the analogous proteins of SBMV and RYMV, CoMV differed from other sobemoviruses in the arrangement and expression of the RDRP and protease ORFs. The CoMV genome organization resembled those of the luteovirus genomes, i.e., the two ORFs overlap out of frame and are hypothesized to yield a frameshift fusion product.

Sobemo- and luteoviruses are two groups of plant viruses characterized by isometric virions containing two positive-strand RNAs of about 4.0 and 1.0 kb that are linked to a VPg (genome-linked protein) and that lack a 3'-terminal poly(A) (12,18,19,20,33). Comparative nucleotide sequence analysis allows the subdivision of luteoviruses into two (28) or three subgroups, all retaining obvious capsid protein homology. However, the amino acid sequences of the subgroup that includes potato leafroll luteovirus (PLRV) and beet western yellows luteovirus (BWYV) bear the closest similarity to those encoded by three sequenced sobemoviruses: southern bean mosaic virus strain C (SBMV-C), SBMV strain B (SBMV-B), and rice yellow mottle virus (RYMV) (13,22,23,37). This finding suggests that some luteoviruses originated by recombination between different virus genomes (21,26). Despite the amino acid sequence conservation, the arrangement and mode of expression of the genes coding for the putative protease and RNA-dependent RNA polymerase (RDRP) differ significantly between the PLRV subgroup of luteoviruses and the sobemoviruses. In luteoviruses, the putative RDRP open reading frame (ORF) is produced as a frameshift fusion protein with the product of the preceding protease ORF (24), whereas in sobemoviruses both genes are translated in the same reading frame as a single polypeptide (37) (Fig. 1).

In this report, we present the nucleotide sequence of the genomic RNA of a sobemovirus isolated from naturally infected cocksfoot plants. Comparison of the gene maps and the encoded amino acid sequences indicates that this virus represents a Russian iso-

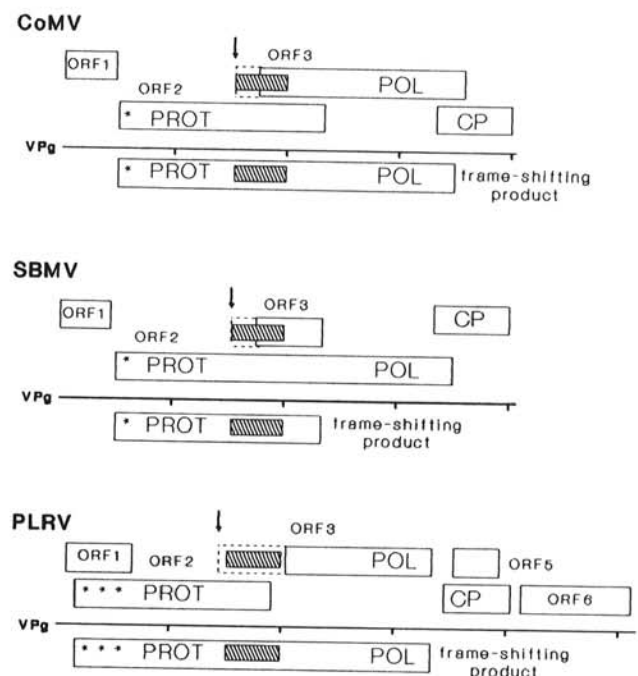


Fig. 1. Schematic representation of cocksfoot mottle sobemovirus, southern bean mosaic sobemovirus (36), and potato leafroll luteovirus (19) genome maps. Open reading frames (ORFs) are shown as rectangles. Regions coding for RNA-dependent RNA polymerase and proteases are indicated as POL and PROT, respectively. The ORFs coding for the coat proteins are marked as CP, and putative transmembrane region locations are marked with asterisks. Arrows indicate the positions of the frameshift sites. Hatched rectangles denote the area of the ORF3 domains. The left sides of the dotted-line rectangles preceding each ORF3 correspond to the position of the last termination codon in the same frame.

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late of a cocksfoot mottle virus (CoMV) and is closely related to a Norwegian isolate of CoMV (17). Our data confirm that CoMV has a unique arrangement of the RDRP and protease ORFs that is more similar to the PLRV subgroup of luteoviruses than to other sobemoviruses.

MATERIALS AND METHODS

Sap transmission and virion RNA isolation. Diseased cocksfoot (*Dactylis glomerata*) leaves were collected in the Moscow region during the fall of 1994. Sap transmission to *Chenopodium amaranticolor*, barley, and wheat plants was carried out with diseased cocksfoot leaf extracts deluted 10-fold in 10 mM phosphate buffer, pH 7.0, as an inoculum. Virus was propagated in barley.

Virus particles were purified by Cs_2SO_4 centrifugation according to the method of Hull (11). Virus RNA was extracted by sodium dodecyl sulfate-phenol-chloroform treatment (38).

Complementary DNA synthesis and cloning. *Escherichia coli* poly(A) polymerase was used to poly(A) tail the RNA. Double-stranded cDNA was synthesized essentially as described by Watson and Jackson (35). The first strand of the cDNA primed with random hexadeoxynucleotides or oligo(dT)₁₂₋₁₈ was synthesized with M-MLV RNA-dependent DNA polymerase (Gibco-BRL, Gaithersburg, MD). Second-strand synthesis was accomplished with *E. coli* DNA polymerase I in the presence of RNase H and *E. coli* DNA ligase. Double-stranded cDNA was blunt-ended by treatment with T4 DNA polymerase, then ligated into *Sma*I-restricted alkaline phosphatase-treated plasmid pGEM3z(f+) (Promega, Madison, WI)

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NATAATAGTGGGAAGAAAGACACACTGTTATCGTTCCCTCCCGAATCAGAGGTTGAGAAGTAGCTTAGATGTGCGAACCTCCCTGGTTTTATAACCGTTCAGTGCTACACCGGCAC 120
                                ORF1  M C E P P P G F I T V Q C Y T S D

GATTTGTTGACAGCGATTCTCAAAATCGTCAAGTCCATTCCCGTCCGCTCGTCTCTCCGCGAAAGCGTTGAAGTTGTTCTGTTCCCGTGTGAGTCTAACAGACATCGCTGTGCGAAG 240
D L L T G D S T I V K S I P V R S C F F R Q G V E V V L F R C E S N R H R W S K

ATCAGAGGTCCTGTGAACCTGACAGTTCATTGTGATATCTGTAATCCCGGAAACTGTTGAGATTCCATCCCTGCCAAAGGCTTTAAAGATATCAAGCGATTCTCTTACAGTGTAAAC 360
I R G P V N L T V H C D I C E F R E T V E I P S L P K G F K V S S D F S Y S V T

TGGAATGTCTACAGCCGTGGCAGGACAGAGTAGCACCGAGGTTAGTTGTTGAGGGTGACCAAGAATGGGTTGTTCTGTTGTTGAAACTGCAAGTCCGTGATGTTGATGAGCAGGA 480
W N C C Y S R G R T E *
                                ORF2  M G C S V V G N C K S V M L M S R M

TGAGTTGGTCAAAAGCTAGCTCTCCCTCGTATCCGTCGCAATGGCGGCGACCCATGACAGACTCCCAACCCACTCTGATATGTATGGGGATCCTAGTAGAGCGTGGTCTCAACTGGATCGTTT 600
S W S K L A L L V S V A M A A A M T D S P P T L I C M G I L V S V V L N W I V C

CGCAGTATCGAAGAGCTTCCGAGCTAATCTGGGGGTTCCCTTGGAAAGCGACTCGTCCCTAGTCCCGGACGGGTTATAGGAGAGCCGGTCTTTGACCCCGATATGGCTATGCTGCTC 720
A V C E E A S E L I L G V S L E A T R P S P G R V I G E P V F D P R Y G Y V A P

CTGCTACTATGATGGCAAGTCGTCGACGCTGATCCCTGCTATCTCCGCTCTGCTCCGCTTCAACAAAGGAAAGGAGACAGTGGAGATGGCGGTTGAGAACTCAGACTACAGCCTTAG 840
A I Y D G K S F D V I L P I S A L S S A S T R K E T V E M A V E N S R L Q P L E

AGAGCAGTCAAAACCGAAGTCCCTGGTGGCTTTGTACTCGCAAGACTGCTATCAGGCTGGGTTCTCGGATTAAGGCGCTGATGGCCAGGAATACCTCTTGACAGCCCTGACGTTGT 960
S S Q T P K S L V A L Y S Q D L L S G W G S R I K G P D G Q E Y L L T A L H V W

GGGAGACTAACATCTCCCATCTTTGTAAGGATGGAAGAAAGTACCAGTAAGCGGATGCCAATCGTTGCAAGCTCCGCTGATCGGACTTGGACTTTGTCTCTGTCGTCGCGAAGA 1080
E T N I S H L C K D G K K V P I S G C P I V A S S A D S D L D F V L V S V P K N

ACGCTTGGTCTGTTTTAGGCGTCGGTGTGGCTGTTGGAATTGCTCAAAACCGAACCCTCGTAACTGCTATGGAGGCTTAGACTCAAAGACCACCTACTGTGCACTGGTGTGGCGG 1200
A W S V L G V G V A R L E L L K R R T V V T V Y G G L D S K T T Y C G T G V A E

AGTTGAAACCCTCCGATATAGTCAAGAAAGTGAACACTACAGGGGGTGGTCAGGCTCTCCACTCTACCACAAGGACGCAATCGTCGGGTTGACTTTGGAGCGAGACCATCTGCGG 1320
L E N P F R I V T K V T T T G G W S G S P L Y H K D A I V G L H L G A R P S A G

GGGTCAATAGAGCGTGTAACTGATGCTAGCCCTCAGGGTTGTCGCGAAATTCGTCAGTGTGAAAATAGTGAATATATCCGACCAAAGCAGTGGACCCGCCGAGACTGTATGCCG 1440
V N R A C N V A M A F R V V R K F V T V E N S E L Y P D Q S S G P A R E L D A E

AGACATACAGGAAAGATTAGAGCAAGAAATGCTTTTACCGAGTACAATATATCTGGCAATTACCGGTGAAAACCTCCGACAGAGAGTGGACTACAGCTGAGGCGTTCGCTGCGAAGAT 1560
T Y T E R L E Q G I A F T E Y N I S G I T V K T S D R E M T T A E A L R V A R Y

ACAAACCTTGGGTGGAGAAAGCATGGGATAGTATGATGACGAGGACACCAGGAAGTGCATCCGCTTTAAACTCCAGCGGGCGGGCTCCCTACGGGCGAGTCCGCCCTTGG 1680
K P L G G G K A W G D S D D E D T Q E T A I R P L N C Q R A G S L R G S P P L A
                                ORF3  R G H P G N C N P A F K L P A G G L P T G Q S A L G

CCAACCTATCAGTACGCGGCTACGCTGCGGTGACGAAAGAAATCATCAATCCGACGGCATGCTTTCCGATCCGCTGGAAGTCAAGTTGCGGTTTAGAGAAGCTGTGTGAGAGC 1800
N L S S T R A T S G V T K E S S I P T A C L S D P L E S R V A G L E K L C A E R
Q L I E Y A G Y V W R D E G I I N S D G M P F R S A G K S S C R F R E A V C R A

GGTTCAGAGAGTGTGAGAGTCTGATGACAGTCCCTGAACCAAGAACTCGCTGCGCAAGCCGCGATCGAAAGCAGAAATCGGATCGCTCTCTTCAAGCCGGAAGGTTTGA 1920
F T E M F E L L S Q S S L N S K N S P G Q A A D R K Q K S D R S S S K P E G L K
V H R D V R A A E S E F P E L K E L A W P S R G S K A E I G S L L F Q A G R F E

AAGAGTCGAAGCGCCGCCAATCTGCAATTGGCAATCACTAACCTCCAAGCCCAGTACCGAGGTCGCGACCCGCTCTGCTTCCGACAGAGAGCCCTGGTGTCTGTAAGACTCTGTCG 2040
E S K R P P I C N W Q S L T S K P S T R G P D P A P A S A E S P G V V K T S S Q
R V E A P A N L Q L A I T N L Q A Q Y P R S R P R S C F R R E P W C R E D F V A

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Fig. 2. Nucleotide sequence of cocksfoot mottle sobemovirus genomic RNA (shown as cDNA) and the amino acid sequences of the major open reading frames (ORFs). The amino acid sequence of each polypeptide with amino acid symbols in 1-letter code is indicated below the first base of each codon within an ORF. The entire ORF3 is translated into amino acids starting at the first sense codon. The consensus heptanucleotide signal for ribosomal frameshifting (UUUAAAC) is underlined.

and transformed into competent *E. coli* XL-1B cells according to Hanahan (10). The insert size of the recombinant cDNA clones was determined by restriction analysis of the plasmid DNAs.

DNA and RNA sequence determination. Double-stranded cDNA inserts in plasmids were sequenced by the dideoxynucleotide chain termination method (30). A nested series of exonuclease III deletions was generated from the original cDNA clones with an Erase-a-Base kit (Promega). More than 96% of the genome sequences were determined on both DNA strands, and about 75% of the sequence was determined by examining two clones covering each region. The 5' terminus of the CoMV RNA was sequenced on RNA template with M-MLV RNA-dependent DNA polymerase (GIBCO) and synthetic primer, 5'-GGTATCAAACCAGTCACTCAC-3', complementary to nucleotides 64 to 81 of the final sequence as described by Fichot (5). The 5' terminus of the subgenomic RNA (sgRNA) was mapped by primer extension with 5'-GGTATCAAACCAGTCACTCAC-3' complementary to nucleotides 3271 to 3297 of the final sequence.

Computer analysis. Multiple alignments of amino acid sequences were performed by the MULTALIN program, version 3.0 (INRA, Toulouse, France). Nucleotide and amino acid sequence analyses were performed by the Gene Pro program, version 4.20 (Riverside Scientific, Seattle). Secondary structure predictions were made with DNASIS software supplied by Pharmacia, Upsala, Sweden.

RESULTS

Sap transmission. Cocksfoot leaves collected in the Moscow region in 1994 had chlorotic streaking. Inoculation of barley and wheat plants with sap prepared from these leaves resulted in stripe mosaics and chlorotic mottle. No infection was found in inoculated *C. amaranticolor* plants. Sobemovirus-like isometric particles (12) were detected by electron microscopy in the sap of inoculated plants (data not shown). However, because we did not have an authentic CoMV isolate for biological and serological com-

Fig. 2. (continued from preceding page)

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AGAAATCGAAAGATCGCGCACTCGGGGAAATCAACCTCAAAGCAAGTCCCGGCGTCCCCCTCCCGGAAATCGGGGTCAGCAACCAACAAAGTAATGTATGTGGCTGGCCCTTGGTGTG 2160
K S K R S R T R G K S T S K Q V P A S P S P K S G S A T N K *
E I E K I A H S G E I N L K A S P G V P L A E I G V S N Q Q V I D V A M P L V C
TGAGGCTGTGGTGAAGGCTCCATGCCTTGGCATCCGTAGACCCGCCAGCAGCTGGTCTCCAGGGAACCTCGTGAAGAGGATTGTGTGATCCGGTTAGACTTTTCGTCAAACA 2280
E A V V E R L H A L A S V D P R Q H D W S P E E L V K R G L C D P V R L E V K Q
GGAAACCCATCTCGACAAAGATGAACAAGGCCCTTTTCGGCTTATCTTCTGCTCGCTTGTAGATCAACTGGTAGAACGCATGCTTTTCGGACCCGAGAACACTACAGAAATTGC 2400
E P H S R Q K I E Q G R F R L I S S V S L V D Q L V E R M L F G P Q N T T E I A
TCTGTGGCATTCTAATCCCTCAAACCAAGGATGGCCCTGTCGAAAGCTAGTCAAGTTCGCTGTTATGGAGGATTGGGCGCGCAAGCAACCAACCCCGGGTGCATGGCAGACAT 2520
L M H S N P S K P G M G L S K A S Q V A L L M E D L A R K H Q T H P G A M A D I
CTCAGGTTTGTAGCTGGCTCCGTTAGGATTGGGAAGTCTGGGCTGACGTATCTATGAGGATTGAGCTAGGCTCGTTCCAGCGTTGATGGCGAAGAGCAGCAATCTCACGGTTTTACTGTTT 2640
S G F D W S V Q D W E L M A D V S M R I E L G S F P A L M A K A A I S R F Y C L
GATGAATGCTACCTTCCAAATGACGAATGAGAGCTCTTGACCCAGAACTGCCTGGACTCATGAAGTCCGGGTCGTACTGTACCTCCAGCTCCCACTCCCGCATTGCGCTCCTTATGGC 2760
M N A T F Q L T N G E L L T Q E L P G L M K S G S Y C T S S S N S R I R C L M A
TGAGCTCATCGGCTCGCTTGGTGTATCGCAATGGGAGATGATCTGTTGAGGATGGGTTAATGATGCCCTTAGGAAATATTCAGCGCTAGGCCATCTCTGTAAGAGTATGAGCGTG 2880
E L I G S P W C I A M G D D S V E G W V N D A P R K Y S A L G H L C K E Y E A C
TCCAGTGTCCCAATGAGATTGAAGGAGGTAGCTTTTCTCCCATCTCATCTCAAAGGTCGAGCGAACTGGAAACGTGGCCCAAGTGTCTGTTTCGATATCTCAGCGACCACA 3000
P V L P N G D L K E V S F C S H L I S K G R A E L E T M P K C L F R Y L S G P H
TGATGTGAAAGCTTGGAGATGAGCTTCTCTCGCCGCTGGGCGCAGATCGTCAGATACCTTTCGCGGATTGGCCGGGCTCCGGAAATGATGGTGAAGAAAGAGCAGCAACGA 3120
D V E S L E M E L S S S R R M G Q I V R Y L R R I G R V S G N D G E E R S S N E
                                ORF4 M V R K G A A T K
AAGCCCGCAACCAAAACCCAGAGCTCAGCAGCAGCCTGGGGCCCGCCGAGGAGCGTGGCCGGTCGATGGAGCCAGTCTCTGACCTTTGAACCCCTCCAGCGCGGTTGGTTCCA 3240
S P A T T K T Q S S A A A W G P P Q E A W P V D G A S L S T F E P S S G G W F H
A P Q Q P K P R A Q Q Q P G G R R R R R G R S M E P V S R P L M P P A A V G S T
CCTGAAGGCTGGTAGAGCAAGACTGTGGTGTAGTACTGGTTGATACCGGTATGATCACCAGTATCTGGGCGGTTTCCAGCGCACAGCTGGGACCAGTACTCCCAAGTTTCCA 3360
L E G W *
L K A G R G K T A G V S D M F D T G M I T S Y L G G F Q R T A G T T D S Q V F I
TTGTGTCAACCGCTGCTTTGGACCGTGTGGAACCATAGCCAAGCGTATCGTGTGGAGACCTAAGCATTGGGAAATCGTGTATCTCCCGAGATGTTCTACTCAGACAGATGGTCCA 3480
V S P A A L D R V G T I A K A Y A L W R P K H W E I V Y L P R C S T Q T D G S I
TTGAAATGGGATTTCTCTAGATTTATGCGGATAGCTCCCAACCAACCCGAACTATGGCTCCTCTACCTCCTTCAACAATTCAAACGTTGGGGAGGTGGGATGGCAGCAGCTTC 3600
E M G F L L D Y A D S V P T N T R T M A S S T S F T T S N V W G G G D G S S L L
TGACAGCTCCATGAAGTCCATGGGCAAGCTGTTACGAGTGTCTTCCCTGTGACGAGTTCGAACAAGTGGTTAAGTTATCTGGAGCACTCCTGAGGAATCGGAGAACGCTCATT 3720
H T S M K S M G N A V T S A L P C D E F S N K M F K L S W S T P E S E N A H L
TGACTGACACCTATGTTCCGGCGGCTTCGTTGGTGGGCTGACTTCCCGGTAGTACGCGTACCAACCTGGTATCTTTGGCTTCGGTCCAGGATCTTGTCTCAAGGGATCGGTTTCCC 3840
T D T Y V P A R F V R S D F P V V T A D Q P G H L M L R S R I L L K G S V S P
CTTCTCAAATCTGTAGTTAAGTACCGCAGGATGGCAACCTGTATAACAGTCCCGCGCTTGGTGGAGTCCGTTTGTCCACTGGAAATCTAACTCAAAGCTTTTGTGAAGATTG 3960
S T N L *
ACGGATTGGTATCATCTCCGTAAGGATACCGACCACTGGCTGGGCTGTCACCCCTCCTTTGTGTCTCAAAACGACGAATCTGGCTCGGCAATGACAGGATCATGGCCGGGCGAGT 4080
GCT
                                4083

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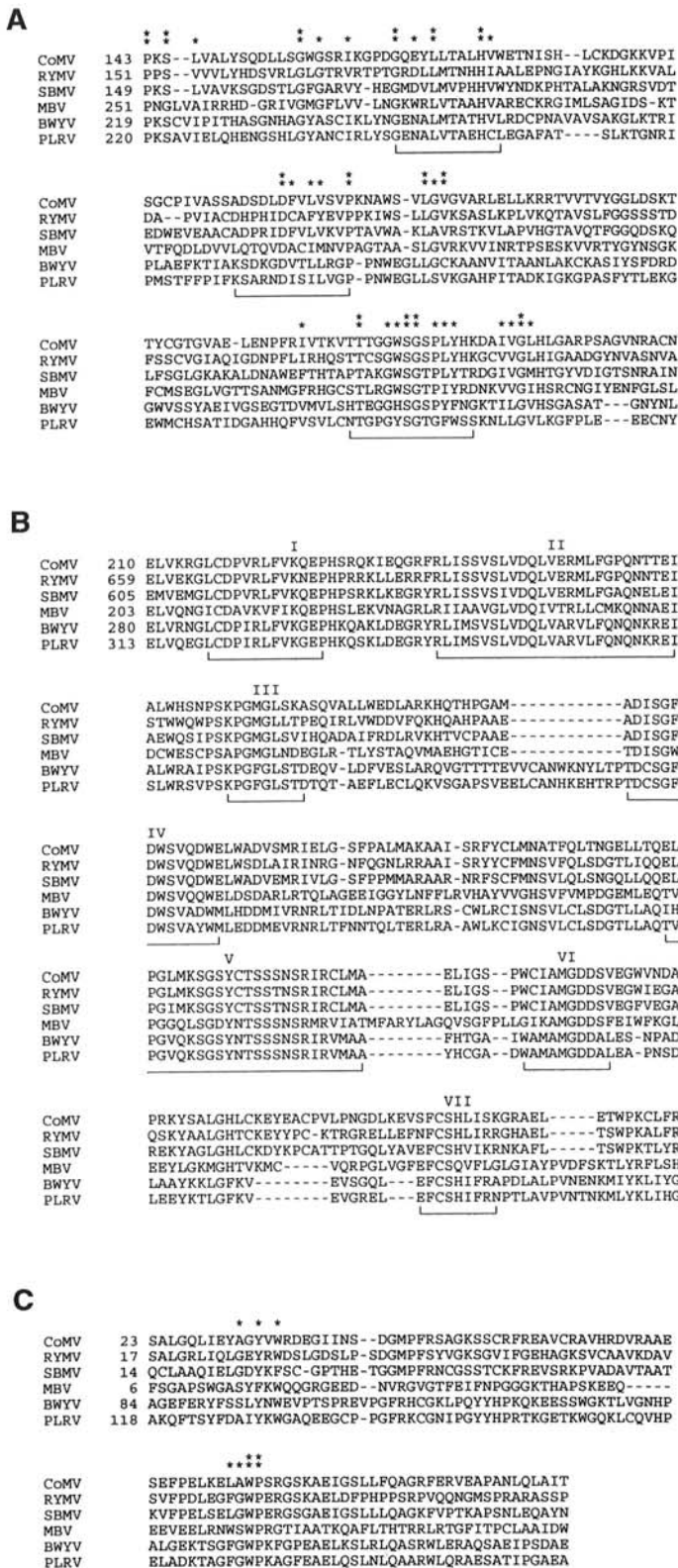


Fig. 3. Sequence alignment of **A**, putative protease, **B**, RNA-dependent RNA polymerase (RDRP), and **C**, ORF3 domains of sobemoviruses (cocksfoot mottle virus [CoMV], rice yellow mottle virus [RYMV], and southern bean mosaic virus [SBMV]), mushroom bacilliform virus (MBV), and luteoviruses (beet western yellows virus [BWYV] and potato leafroll luteovirus [PLRV]). The conserved motifs of protease and RDRP domains, according to Koonin and Dolja (14), are underlined. Double asterisks indicate similar residues; single asterisks mark homologous residues (grouped in accordance to Dolja and Koonin [3]). Gaps are introduced for better alignment. Numbers indicate the distance from the first amino acid of the ORF2-encoded polypeptide (**A**), for southern bean mosaic sobemovirus and rice yellow mottle sobemovirus (**C**), or the position of the first aligned amino acid in the polypeptide starting after the last terminator codon preceding ORF3 in the same frame (**B** and **C**).

parisons with our spherical virus, the alternative means of identifying it was to determine the organization of its genome.

Coding capacity of the virus genome. Analysis of virion RNA of the putative CoMV in 1.0% agarose gel revealed genomic RNA of about 4.0 kb and additional RNA of about 1 kb (data not shown). Encapsulation of the sgRNA is a characteristic feature of sobemoviruses (12).

The complete nucleotide sequence (4083 nt) of CoMV RNA revealed four major ORFs potentially coding for proteins that exceed 100 amino acid residues (Figs. 1 and 2). ORF1 (positions 70 to 396) encodes a 12.3-kDa protein (109 residues). ORFs 2, 3, and 4 overlap and code for proteins of 60.7 (569 residues), 56.3 (505 residues), and 27.6 kDa (255 residues), respectively. CoMV RNA is slightly shorter than SBMV-C RNA (4194 nt), SBMV-B RNA (4109 nt), and RYMV RNA (4450 nt) (22,23,37).

Protein sequence comparisons. ORF1 of CoMV encodes a 12.3-kDa protein (Figs. 1 and 2) that is a highly hydrophilic protein with a neutral net charge. We found no statistically significant similarity between the ORF1 protein and ORF1 proteins of other sobemo- and luteoviruses. Among luteoviruses, the similarities between ORF1 proteins also show only marginal statistical significance (9). The actual function of the ORF1 protein is unknown, and its extreme variability does not allow any reasonable functional predictions.

The CoMV ORF2 protein is predicted to contain at least three distinct domains. Hydrophobicity prediction methods (31) demonstrate that the N-terminal 60 amino acids of the ORF2 protein include two stretches of hydrophobic residues that may form a transmembrane domain. The central part of the CoMV ORF2 product with approximate coordinates 130-140 to 310-330 represents a domain with a motif typical of chymotrypsin-like serine proteases (7,14) found in sobemoviruses and the PLRV subgroup of luteoviruses (Fig. 3A). Based on VPg-containing picornaviruses and plant potyviruses, one can speculate that the hydrophilic protein segment of approximately 70 to 80 amino acids located in the sobemo- and luteovirus ORF2 products between hydrophobic and putative protease domains may represent the viral VPg (1,22,28).

The C-terminal region of ORF3 is predicted (Figs. 1 and 2) to encode a viral RDRP based on the presence of the GDD motif and surrounding conserved motifs characteristic of RNA polymerases (13,14) (Fig. 3C). This protein domain in CoMV shows extensive similarity to the putative RDRPs of sobemoviruses (SBMV-C, SBMV-D, and RYMV), luteoviruses (PLRV, BWYV, barley yellow dwarf virus RPV strain, and cucurbit aphid-borne yellows virus) (9,34), and an unclassified mushroom bacilliform virus (MBV)

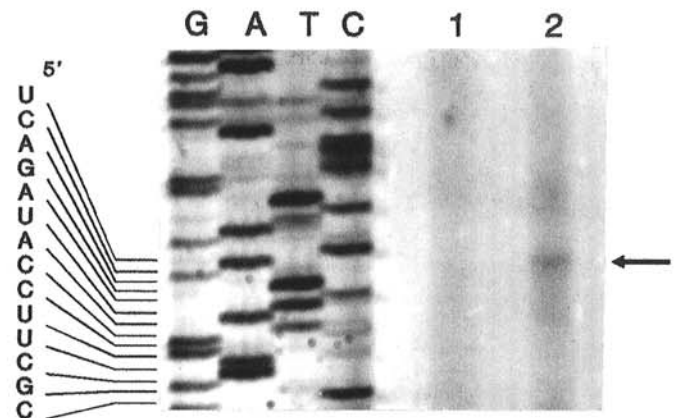


Fig. 4. Mapping of the 5' end of cocksfoot mottle sobemovirus subgenomic RNA (sgRNA). Sequencing of the cDNA clone containing the region of the genomic RNA upstream of the coat protein gene is shown in parallel with the primer extension analysis of genomic RNA (column 1) and sgRNA purified from agarose gel (column 2). The arrow indicates the position of the primer extension product.

(27) (Fig. 3C). These similarities can be extended to include pea enation mosaic virus RNA1 (PEMV) (1) (data not shown).

The arrangement of the ORFs encoding the putative protease and RDRP in the CoMV genome, both in the Russian and Norwegian isolates (17; this paper), is more similar to that in PLRV and BWYV genomes than to the genomes of other sobemoviruses. A consensus heptanucleotide signal (UUUAAAC) for ribosomal frameshifting (underlined in Fig. 2) followed by a stem-loop structure is located in the genomes of both CoMV isolates 108 nucleotides upstream of the initiation codon of ORF3. Thus, ORF2- and ORF3-encoded protease and RDRP of CoMV can be expressed by a -1 translational frameshift as a single polyprotein (17).

The extreme N-terminal region of the sobemovirus ORF3 putative protein exhibits a limited similarity to a domain in the luteovirus ORF2-ORF3 fusion protein encoded approximately 300 to 400 nt downstream of the frameshift point (Figs. 1 and 3C) (17). A computer search detected the counterparts of this protein domain (ORF3 domain) in the ORF3-encoded polypeptides of SBMV and RYMV, as well as in the N-terminal portion of the MBV (Fig. 3C) and PEMV ORF3-encoded product (1). Significantly, some similarities to the CoMV N-terminal ORF3 product domain extend into the protein segments encoded by the SBMV and RYMV ORF3 region located upstream of the ORF3 AUG codon (Fig. 3C).

The CoMV ORF4 located at the 3'-terminal portion of the CoMV genomic RNA coded for the coat protein. The molecular mass of the predicted protein (28 kDa) encoded by ORF4 is similar to that obtained by polyacrylamide gel electrophoresis analysis of the proteins in a purified virion preparation. Comparison of the ORF4-coded protein and the coat proteins of sobemoviruses and some other small spherical plant viruses reveals their obvious sequence similarity (data not shown) (3).

Analysis of the virion-derived coat protein sgRNA. For SBMV, sgRNA of about 1.0 kb has been detected in virions (6). In the CoMV RNA preparation, we (this paper) and others (17) detected a similar RNA by agarose gel electrophoresis. To characterize the CoMV coat-protein sgRNA further, we mapped its 5' terminus relative to the virus genome. A primer complementary to the 5'-terminal segment of the coat protein gene (positions 3271 to 3297) was annealed to gel-purified sgRNA, and the RNA-DNA hybrid was extended with reverse transcriptase. The major primer extension product occurred at position 3057, as revealed by comparison with the sequencing ladder produced from one of the cDNA clones covering this region of the CoMV genome (Fig. 4). In controlled primer extension reaction with isolated genomic RNA, we found no such product.

Thus, the start position of the CoMV sgRNA was located 35 bases upstream of the first AUG codon of ORF4. This initiation codon is in suboptimal context (GAAAUGA) (15) and is followed tandemly by the second AUG, which is in an optimal context (AUGAUGG) (Fig. 5). Comparison of the nucleotide sequences upstream of the coat protein genes of sobemoviruses suggests the role of the sequence UCAGA or its variant, UCGAA, in the generation of coat-protein sgRNAs (Fig. 5). Regions preceding the start sites of sgRNAs in positive-sense viral RNAs may include the core elements of subgenomic promoters (4). Within a particular virus group, these regions usually have common sequence motifs (2,8,32,36). Unexpectedly, we found no extended common sequence motifs located 5' of the UCAGA sequence in sobemovirus genomes.

However, comparison of the sequences downstream of the UCAGA in different sobemovirus genomes (Fig. 5) indicates that all virus RNAs include GC-rich inverted repeats potentially forming 5- or 6-bp stem structures with a terminal loop of 10 to 12 nt. These stem-loop structures exhibit a high level of sequence conservation (particularly, all loops have a CCN₁UC motif) and may play a role in the generation of sgRNA as *cis*-acting elements recognized by RDRP.

DISCUSSION

The isometric virus isolated from cocksfoot plants with chlorotic streaking symptoms was identified as a Russian isolate of CoMV. This conclusion is based mainly on viral nucleotide and encoded polypeptide sequence comparisons. It is obvious that the sequenced genome represents a true sobemovirus genome that has, however, some unique properties. The major difference in the CoMV genome structure compared with those of other sobemoviruses is the arrangement of the putative RDRP and protease genes (17). These genes, like those in luteovirus genomes, overlap in different reading frames in contrast to other known sobemovirus genomes that harbor these genes in a single translation unit.

The nucleotide sequence of a Norwegian isolate of the CoMV genomic RNA (17) shows over 96% identity to the Russian isolate of CoMV at the nucleotide level. A similar level of identity

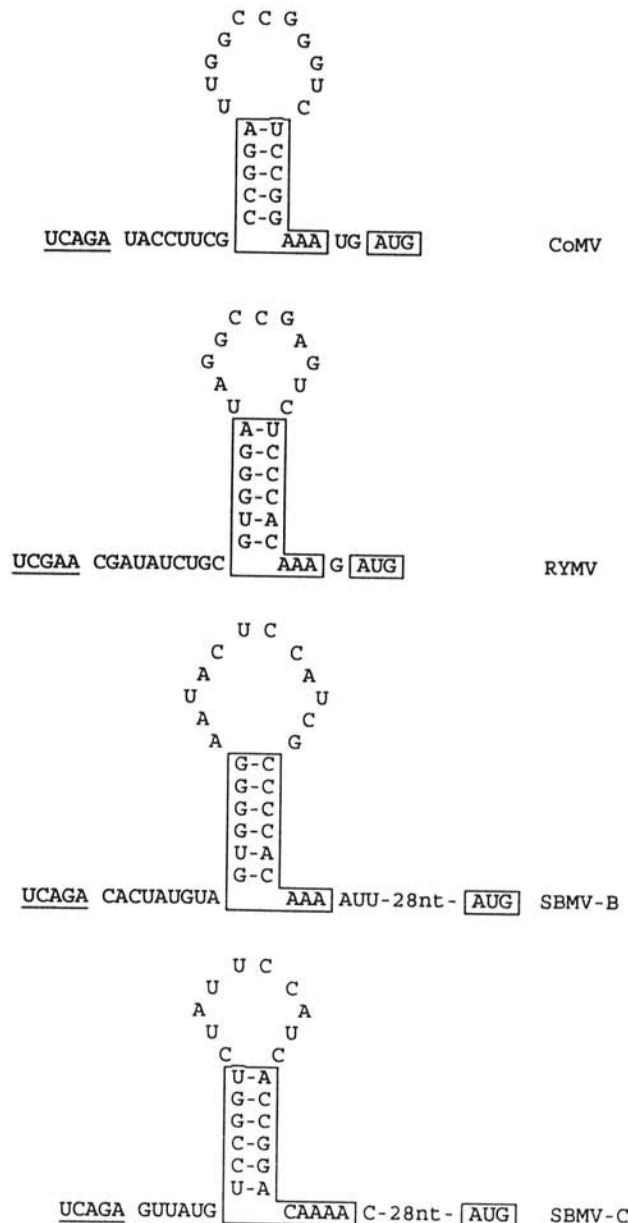


Fig. 5. The predicted stem-loop structures of the mapped 5' end of the cocksfoot mottle sobemovirus (CoMV) coat protein subgenomic RNA (sgRNA) and the putative 5' ends of the coat protein sgRNAs of other sobemoviruses (rice yellow mottle virus [RYMV], southern bean mosaic virus strain C [SBMV-C], and SBMV strain B [SBMV-B]). The conserved 5'-terminal UCAGA sequence (UCGAA in RYMV) is underlined. The conserved regions of the RNA secondary structure and coat protein gene initiation codons are boxed.

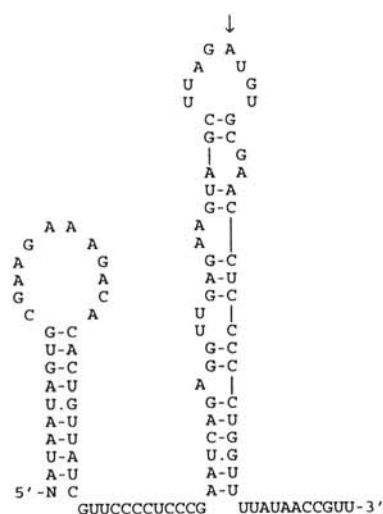
was revealed in comparisons of the nucleotide sequences of different strains of potato virus X (25,32). Experimental studies on the genome expression strategy of the Norwegian strain of CoMV have shown that the ORF3-coded protein (ORF2b as named by the authors) is synthesized as part of the polyprotein, which includes an ORF2 protein (ORF2a), by -1 ribosomal frameshifting (16). This mode of expression is strongly reminiscent of that found in luteovirus genomes (24). One may speculate that in the course of the expression of SBMV and RYMV nonstructural proteins there is also a translational frameshift, e.g., the ORF3 protein is translated as a fusion with the N-terminal half of ORF2 (16) (Fig. 1). Significantly, in this respect both SBMV and RYMV genomes have a consensus signal for ribosomal frameshift, including a shifty heptanucleotide immediately followed by a stem-loop structure at the beginning of the overlapping region between ORF2 and -3 (16). Thus, despite the differences in expression mode of RDRP, all sobemoviruses can express some of the protein products (ORF3 domain) solely by translational frameshift-

ing either as a polyprotein with N-terminal hydrophobic and protease domains (SBMV and RYMV) or as a longer polyprotein with an additional C-terminal RDRP domain (CoMV).

The 5'- and 3'-untranslated regions (UTRs) in the CoMV genome are 69 and 228 nt, respectively. The 5' UTR in CoMV RNA has a sequence similar to those of other sobemoviruses (17). In RYMV RNA, computer-assisted folding of the 60 most 5'-proximal nucleotides shows a stable stem-loop structure similar to that of the 5' UTR of the chloroplast *psbA* mRNA (22). A similar type of folding may be predicted for the 30 most 5' residues in CoMV RNA (Fig. 6A). Interestingly, the loop sequence GAAAG in the RYMV RNA 5'-terminal hairpin structure also occurs in the center of that this structure in the CoMV most 5' hairpin (Fig. 6A). The importance of the 5'-terminal stem-loop structures as *cis*-acting signals for (+)RNA synthesis has been demonstrated for the genomes of different plant positive-strand RNA viruses (4).

The 3' UTRs in the genomic RNAs of sobemoviruses show only marginal sequence conservation. However, in CoMV RNA, as in SBMV and RYMV genomic RNAs (22), the extreme 3'-terminal region can form an extended stem-loop structure that contains tRNA-like motifs (Fig. 6B). It has been suggested that the 3'-terminal hairpins, particularly the tRNA-like clover-leaf structure, may act as *cis* elements in genomic promoter regions of some VPG-containing RNA viruses (4,29). In line with this, we have identified a clover-leaf structure within 130 3'-terminal nt of CoMV RNA (Fig. 6B).

A



B

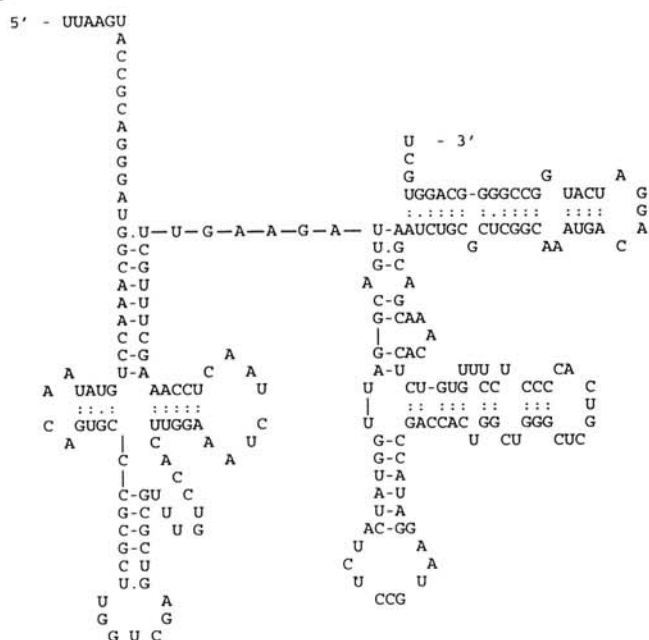


Fig. 6. The predicted stem-loop structures of the A, 5'- and B, 3'-untranslated regions of cocksfoot mottle virus RNA. The ORF1 initiation codon is underlined.

LITERATURE CITED

- Demler, S. A., and de Zoeten, G. A. 1991 The nucleotide sequence and luteovirus-like nature of RNA1 of an aphid non-transmissible strain of pea enation mosaic virus. *J. Gen. Virol.* 72:1819-1834.
- Ding, S., Howe, J., Keese, P., Mackenzie, A., Meek, D., Osorio-Keese, M., Skotnicki, N., Srifah, P., Torronen, M., and Gibbs, A. 1990. The tobex, a sequence shared by most tobamoviruses: Its use in molecular studies of tobamoviruses. *Nucleic Acids Res.* 18:1181-1187.
- Dolja, V. V., and Koonin, E. V. 1991. Phylogeny of capsid proteins of small icosahedral RNA plant viruses. *J. Gen. Virol.* 72:1481-1486.
- Duggal, R., Lahser, F. C., and Hall, T. C. 1994. *Cis*-acting sequences in the replication of plant viruses with plus-sense RNA genomes. *Annu. Rev. Phytopathol.* 32:287-309.
- Fichot, O., and Girard, M. 1990. An improved method for sequencing of RNA template. *Nucleic Acids Res.* 18:6162.
- Ghosh, A., Rutgers, T., Mang, K. Q., and Kaesberg, P. 1989. Characterization of the coat protein mRNA of southern bean mosaic virus and its relationship to the genomic RNA. *J. Virol.* 39:87-92.
- Gorbalenya, A. E., Donchenko, A. P., Blinov, V. M., and Koonin, E. V. 1989. Cystein proteases of positive-strand RNA viruses and chymotrypsin-like serine proteases. A distinct protein superfamily with a common structural fold. *FEBS Lett.* 243:103-114.
- Goulden, M. G., Lomonosoff, G. P., Davies, J. W., and Wood, K. R. 1990. The complete nucleotide sequence of PEBV RNA2 reveals the presence of a novel open reading frame and provides insights into the structure of tobamovirus subgenomic promoters. *Nucleic Acids Res.* 18:4507-4512.
- Guilley, H., Wipf-Scheibel, C., Richards, K., Lecoq, H., and Jonard, G. 1994. Nucleotide sequence of Cucurbit aphid-borne yellow luteovirus. *Virology* 202:1012-1017.
- Hanahan, D. 1986. Techniques for transformation of *E. coli*. Pages 109-135 in: *DNA Cloning: A Practical Approach*. Vol. 1. D. M. Glover, ed. IRL Press Limited, Oxford.
- Hull, R. 1977. The banding behaviour of the viruses of the southern bean mosaic virus group in gradients of caesium sulphate. *Virology* 79:50-57.
- Hull, R. 1988. Southern bean mosaic virus. Pages 113-146 in: *The Plant Viruses*, vol. 4. R. Francki, ed. Plenum, New York.
- Koonin, E. V. 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *J. Gen. Virol.* 72:2197-2206.
- Koonin, E. V., and Dolja, V. V. 1993. Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* 28:375-430.
- Kozak, M. 1989. A scanning model for translation: An update. *J. Cell Biol.* 108:229-241.
- Makinen, K., Naess, V., Tamm, T., Truve, E., Aaspollu, A., and Saarma, M. 1995. The putative replicase of the cocksfoot mottle sobemovirus is translated as a part of the polyprotein by -1 ribosomal frameshift. *Vir-*

- ology 207:566-571.
17. Makinen, K., Tamm, T., Naess, V., Truve, E., Puurand, C., Munthe, T., and Saarma, M. 1995. Characterization of cocksfoot mottle sobemovirus genomic RNA and its sequence comparison with related viruses. *J. Gen. Virol.* 76:2817-2825.
 18. Mang, K. Q., Ghosh, A., and Kaesberg, P. 1982. A comparative study of the cowpea and bean strains of southern bean mosaic virus. *Virology* 116:264-274.
 19. Mayo, M. A., Barker, H., Robinson, O. J., Tamada, T., and Harrison, B. D. 1982. Evidence that potato leafroll virus is positive-stranded is linked to a small protein and does not contain polyadenylate. *J. Gen. Virol.* 59:163-167.
 20. Mayo, M. A., Robinson, D. J., Jolly, C. A., and Hyman, L. 1989. Nucleotide sequence of potato leafroll luteovirus RNA. *J. Gen. Virol.* 70:1037-1051.
 21. Miller, W. A., Dinesh-Kumar, S. P., and Paul, C. P. 1995. Luteovirus gene expression. *Crit. Rev. Plant Sci.* 14:179-211.
 22. Ngon A Yassi, M., Ritzenthaler, C., Brugidon, C., Fauquet, C., and Beachy, C. F. 1994. Nucleotide sequence and genome characterization of rice yellow mottle virus RNA. *J. Gen. Virol.* 75:249-257.
 23. Othman, Y., and Hull, R. 1995. Nucleotide sequence of bean strain of southern bean mosaic virus. *Virology* 206:287-297.
 24. Pruffer, D., Tacke, E., Schmitz, J., Kull, B., Kuafmann, A., and Rohde, W. 1992. Ribosomal frameshifting in plants: A novel signal directs the -1 frameshift in the synthesis of the putative viral replicase of potato leafroll virus. *EMBO J.* 11:1111-1117.
 25. Querci, M., van der Vlugt, R., Goldbach, R., and Salazar, L. F. 1993. RNA sequence of potato virus X Strain H B. *J. Gen. Virol.* 74:2251-2255.
 26. Rathjen, J. P., Karegeorgos, R. E., Habili, N., Waterhouse, P. M., and Symons, R. H. 1994. Soybean dwarf luteovirus contains the third variant genome type in the luteovirus group. *Virology* 198:671-679.
 27. Revill, P. A., Davidson, A. D., and Wright, P. J. 1994. The nucleotide sequence and genome organization of mushroom bacilliform virus: A single-stranded RNA virus of *Agaricus bisporus* (Lange) Imbach. *Virology* 202:904-911.
 28. Riechmann, J. L., Lain, S., and Garcia, J. A. 1992. Highlights and prospects of potyvirus molecular biology. *J. Gen. Virol.* 73:1-16.
 29. Rohll, J. B., Holness, C. L., Lomonossoff, G. P., and Manle, A. J. 1993. 3'-Terminal nucleotide sequences important for the accumulation of cowpea mosaic virus M-RNA. *Virology* 193:672-679.
 30. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
 31. Sipos, L., and Von Heijne, G. 1993. Predicting the topology of the eukaryotic membrane proteins. *Eur. J. Biochem.* 213:1333-1340.
 32. Skryabin, K. G., Morozov, S. Yu., Kraev, A. S., Rosanov, M. N., Chernov B. K., Lukasheva, L. I., and Atabekov, J. G. 1988. Conserved and variable elements in RNA genomes of potexviruses. *FEBS Lett.* 204:33-40.
 33. Veidt, I., Lot, H., Leiser, M., Scheidecker, D., Guilley, H., Richards, K., and Jonard, G. 1988. Nucleotide sequence of beet western yellows virus RNA. *Nucleic Acids Res.* 16:9917-9932.
 34. Vincent, J. R., Lister, R. M., and Larkins, B. A. 1991. Nucleotide sequence analysis and genome organization of the NY-RPV isolate of barley yellow dwarf virus. *J. Gen. Virol.* 72:2347-2355.
 35. Watson, C. J., and Jackson, J. F. 1986. An alternative procedure for the synthesis of double-stranded cDNA for cloning in phage and plasmid vectors. Pages 79-88 in: *DNA Cloning: A Practical Approach*. Vol. 1. D. M. Glover, ed. IRL Press Limited, Oxford.
 36. White, K. A., Bancroft, J. B., and Mackie, G. A. 1992. Mutagenesis of a hexanucleotide sequence conserved in potexvirus RNAs. *Virology* 189:817-820.
 37. Wu, S., Rinehart, C. A., and Kaesberg, P. 1987. Sequence and organization of Southern bean mosaic virus genomic RNA. *Virology* 161:73-80.
 38. Zimmer, D. 1975. The 5' end group of tobacco mosaic virus RNA is m7GpppGp. *Nucleic Acids Res.* 2:1189-1201.