Complete Nucleotide Sequence of *Ryegrass Mottle Virus* : A New Species of the Genus *Sobemovirus*[†]

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ABSTRACT

The genome of *Ryegrass mottle virus* (RGMoV) comprises 4210 nucleotides. The genomic RNA contains four open reading frames (ORFs). The largest ORF 2 encodes a polyprotein of 947 amino acids (103.6 kDa), which codes for a serine protease and an RNA-dependent RNA polymerase. The viral coat protein is encoded on ORF 4 present at the 3'-proximal region. Other ORFs 1 and 3 encode the predicted 14.6 kDa and 19.8 kDa proteins of unknown function. The consensus signal for frameshifting, heptanucleotide UUUAAC and a stem-loop structure just downstream is in front of the AUG codon of ORF 3. Analysis of the *in vitro* translation products of RGMoV RNA suggests that the 68 kDa protein may represent a fusion protein of ORF 2-ORF 3 produced by frameshifting. The protease region of the polyprotein and coat protein have a low similarity with that of the sobemoviruses (approximately 25% amino acid identity), while the RNA-dependent RNA polymerase region has particularly strong similarity (54 to 60% of more than 350 amino acid residues). The sequence similarities of RGMoV to the sobemoviruses, together with the characteristic genome organization indicate that RGMoV is a new species of the genus *Sobemovirus*.

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Key words : *Ryegrass mottle virus*, nucleotide sequence, genome organization, sobemovirus.

INTRODUCTION

Ryegrass mottle virus (RGMoV) was first isolated from stunted Italian ryegrass (*Lolium multiflorum*) and cocksfoot (*Dactylis glomerata*) having mottling and necrotic symptoms on leaves²⁰. The isometric particle, 28 nm in diameter contains a species of single-stranded RNA with a molecular weight of 1.5×10^6 . The physical properties and some biological ones of the virus are similar to *Cocksfoot mottle virus* (CfMV), which is prevalent in cocksfoot pastures in Japan¹⁹. However, RGMoV is serologically distinct from CfMV, *Cocksfoot mild mosaic virus*, *Cynosurus mottle virus* and *Phleum mottle virus*, which occur in European countries²⁰. Last year, an isometric virus, isolated from Italian ryegrass in Germany was found to be serologically related to RGMoV ; in agar gel double diffusion tests, a spur formed between RGMoV and the Germany isolate (Frank Rabenstein, Germany ; personal communication).

In spite of serological differences between RGMoV

and sobemoviruses²⁰, general properties of RGMoV are similar to those of grass viruses that belong to sobemoviruses⁴. The genome sequence of sobemoviruses has been determined in *Southern bean mosaic virus* (SBMV)^{12,24}, CfMV^{8,15}, *Rice yellow mottle virus* (RYMV)¹¹ and *Lucerne transient streak virus* (LTSV, accession number U31286). The genomic RNA of sobemoviruses is a single-stranded molecule, approximately 4100 to 4500 nucleotides (nt) in size. The 5' terminus has a genome-linked viral protein (VPg) and the 3' end does not have a poly (A) tail. The genome encodes four ORFs : the largest ORF encodes the polyprotein of approximately 100 kDa, which contains protease and RNA polymerase motifs. Only the polyprotein of CfMV is encoded by two smaller overlapping ORFs, by -1 frame shifting⁷. Recently, we determined the complete nucleotide sequence of the Japanese isolate of CfMV (CfMV/JP) (Zhang and Toriyama, unpublished data ; accession number AB040447). The nucleotide sequence is 96.8% identical to the Norwegian isolate of CfMV (CfMV/NO)⁸ and 95.8% identical to the Russian isolate¹⁵. Its

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genome organization is identical to that of CfMV. So far, the genome sequence of RGMoV and the Germany isolate has not been determined, so the genus is still unknown. In this paper, we report the complete nucleotide sequence of RGMoV and compare it to that of the sobemoviruses.

MATERIALS AND METHODS

Viruses *Ryegrass mottle virus* (RGMoV) was propagated in barley plants (cv. Shunsei) and purified as described previously²⁰. A purified preparation of CfMV/JP, was stored at -80°C ¹⁹ and used for the *in vitro* translation experiment.

Terminal sequence of viral RNA In a preliminary experiment, we found that RGMoV RNA does not have a poly (A) tail at the 3'-terminus. Thus, we determined the 3'-terminal sequence by two-dimensional mobility shift analysis as described previously¹⁷. In this experiment, the homomix (alkaline digested yeast RNA mixture) was prepared by using the RNA from *Torula utilis*, a product of Fluka (Riedel-de Haën; Seelze, Germany). The 5'-terminal sequence of RGMoV RNA was identified by sequencing the PCR clones amplified by using the 5' RACE abridged anchor primer system (Gibco BRL, Gaithersburg, USA).

Cloning and DNA sequence cDNA synthesis was done as described previously²¹ using M-MLV reverse transcriptase (Gibco BRL), random hexanucleotide primer and synthetic oligonucleotide primer (P1), 5'-ACTAGTCGACACGAAAACCCC-3': the sequence at the 3' end underlined was analyzed by two-dimensional sequence analysis. The synthesized second strand cDNA was blunt-ended with T4 DNA polymerase and ligated into *Sma*I-digested pUC18. Recombinant plasmids were transformed into competent *Escherichia coli* DH5 α (TOYOBO, Osaka, Japan). The cDNA clones shown in

Fig. 1 were made by primer extension and PCR amplification and used for sequencing of RGMoV RNA. The ambiguous nucleotide sequence was confirmed by using PCR clones prepared independently (not shown in Fig. 1). Nucleotide sequences were determined using the Pharmacia DNA sequencing kit and an ALFred DNA sequencer (Pharmacia, Uppsala, Sweden). The sequence data were assembled and analyzed using the DNASIS (Macintosh) program (Hitachi Software Engineering Co., Yokohama, Japan). GeneBank/EMBL, NBRF and PIR databases were searched for nucleic acid and amino acid sequence identity.

Cell-free translation *In vitro* translation using wheat germ extract (Promega, Madison, USA) was performed as described by the manufacturer's manual in a final volume of 50 μl in the presence of redive L-[^{35}S] methionine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 hr at 25°C . Translation products were separated by SDS-PAGE (10% polyacrylamide) and detected using a Molecular Imager System (BioRad, Richmond, USA). A set of prestained SDS-PAGE standards (BioRad) was used as protein size markers.

N-terminal amino acid sequence Purified RGMoV was electrophoresed on 10% polyacrylamide-SDS gels and electro-blotted onto the PVDF membrane (Immobilon-P^{sq}; Millipore, Middlesex, UK). The portion corresponding to the coat protein on the PVDF membrane was excised, and the N-terminal sequence of the coat protein was analyzed using a gas-phase protein sequencer (model 477A/120A, Applied Biosystems, Foster City, USA).

RESULTS AND DISCUSSION

Nucleotide sequence and genome organization

The complete nucleotide sequence of RGMoV com-

Fig. 1. cDNA clones used to determine the nucleotide sequence of RGMoV RNA. Solid lines with each number, cDNA clones synthesized using synthetic primer P1 or by primer extension; Slant lines, PCR clones.

Fig. 2. The ORF maps on the genome of RGMoV, SBMV, RYMV and CfMV/JP. The ORFs are illustrated as boxes. polypr, polyprotein ; CP, coat protein ; arrows, positions of frameshifting sites. References of sequence data : SBMV (M23021), RYMV (L20893), CfMV/JP (AB040447) and CfMV/NO (Z48630).

Table 1. Amino acid sequence similarity between coding regions of RGMoV and sobemoviruses

Viruses ^{a)}	ORF 1	ORF 2 : polyprotein		ORF 3	ORF 4 : coat protein
		Protease region	Polymerase region		
SBMV	31.6% (38 aa) ^{b)}	26.0% (346 aa)	54.2% (369 aa)	42.1% (122 aa)	26.8% (157 aa)
LTSV	29.0 (62)	29.7 (353)	56.0 (357)	40.3 (120)	25.3 (154)
RYMV	26.7 (15)	20.1 (259)	54.2 (331)	34.3 (48)	23.8 (67)
CfMV	26.3 (19)	23.4 (397)	59.0 (332)	48.3 (89) ^{c)}	14.9 (210)

a) References of sequence data : SBMV (M23021), LTSV (U31286), RYMV(L20893) and CfMV (Z48630).

b) The percentage values indicate the identity over the stretch of amino acid residues indicated in parentheses.

c) This similarity was found between the N-terminal region of the 56.3 K ORF of CfMV (refer to Fig. 2).

prises 4210 nt with a base composition of 24.3% A, 22.2% U, 25.2% C and 28.3% G. The G+C content is 53.5%. The sequence contains four major ORFs flanked by 5'- and 3'-untranslated sequences of 99 and 198 nt, respectively. Database searches indicated that the genome sequence of RGMoV is significantly similar to that of sobemoviruses, for which the genome organization is summarized in Fig. 2. As shown in Fig. 2, ORFs 1 and 2 of RGMoV are separated by the intergenic region of 143 nt, but other ORFs overlap each other. RGMoV ORF 1 encodes a predicted 14.6 kDa protein of 133 amino acids. No distinct sequence similarity was found between RGMoV and the corresponding ORF 1 of sobemoviruses (Table 1). The

ORF 1 of RYMV/ Ivory Coast isolate (P1 protein) is required for viral replication and virus spread¹¹. So far the function of the 14.6 kDa protein of RGMoV is unknown.

The largest ORF 2 extends from nucleotides 643 to 3486. The predicted 103.6 kDa protein consists of 947 amino acids. Database searches revealed a significant similarity to the polyproteins of sobemoviruses ; SBMV (accession number, M23021)²⁴, RYMV (L20893)¹¹, CfMV (Z48630)⁸ and LTSV (U31286). The polyprotein of RGMoV contains serine and P3C proteases^{3,25} and an RNA-dependent RNA polymerase¹³ (Fig. 3). A conserved sequence, GxPxFDPxYG⁸, is found in the N-terminal

Fig. 3. Schematic representation of RGMoV genome organization. The ORFs are shown as boxes on the genomic RNA (a line with vertical bars, 1000 nt scale). PRO, region coding for serine protease and P3C protease; VPg, a putative region for genome-linked viral protein. Putative E/S cleavage sites around the VPg are shown with vertical lines; POL, region for RNA-dependent RNA polymerase; CP, ORF coding for RGMoV coat protein; F/S with an arrow indicates the characteristic frameshifting site. Shaded box under F/S represents the putative ORF 2-ORF 3 transframe fusion product.

region (amino acids 70 to 90 residues) of the 103.6 kDa polypeptide. The protease motif appears immediately downstream of the conserved sequence: serine protease, in amino acids 148 to 220 from N-terminus and P3C protease, in amino acids 272 to 300 (Fig. 3). The serine protease motif is well conserved between RGMoV, sobemoviruses and poliovirus^{3,25}. In addition, the P3C protease motif $\cdots \text{xGxS}^*/\text{C}^*\text{GxxxxxxxGxxxxGxH}^*\cdots$ (the catalytic amino acid residue is marked with asterisks), is present just downstream. However, instead of serine (S*) or cysteine (C*), alanine is found in RGMoV. Thus, it is uncertain whether the P3C protease domain is catalytic in RGMoV or not.

The RNA-dependent RNA polymerase is encoded near the C-terminal region of the polypeptide. This region showed very strong similarity, 54 to 60% identity over a 350 amino acid stretch (Table 1). The RNA polymerase motifs¹³ are distributed between amino acids 680 to 810. The sequence of this domain is conserved in particular, with approximately 75% identity between RGMoV and sobemoviruses. Database searches also showed that the sequence of RGMoV polymerase is highly conserved between the RNA polymerases of *Beet mild yellowing virus* (S65829), *Cucurbit aphid-borne yellowing virus* (X76931), *Potato leaf roll virus* (X74789) and *Barley yellow dwarf virus* (L25299) of the family *Luteoviridae*. The similarity is approximately 50% identity over a 240 amino acid stretch, suggesting an evolutionary close relationships between RGMoV, sobemovirus and luteovirus (subgroup II)¹⁰.

Van der Wilk *et al.*²² found that the VPg of SBMV is encoded by ORF 2, downstream of the protease domain and in front of the RNA polymerase. We compared the amino acid sequence similarity between the VPg region of SBMV ORF 2 and the corresponding region of RGMoV ORF 2. The search revealed no significant similarity. Sequence diversities in the VPg region²² are also shown between SBMV, CfMV and RYMV. However, the con-

served sequence, WAG + E/D rich sequence is detected in the region, and putative E/S cleavage sites are present on both sides of the region: proteolytic cleavage would result in a protein of 9 kDa. Possibly, the VPg of RGMoV is located between the protease and the RNA-dependent RNA polymerase domains in the same order as in the SBMV ORF 2²² (Fig. 3).

RGMoV ORF 3 is completely within the ORF 2. The predicted 19.8 kDa protein has distinct similarity, 40% identity to the corresponding ORFs of SBMV and LTSV. However, it is unknown whether the 19.8 kDa protein is independently translated *in vivo*, because ORF 3 may be expressed as a fusion protein as will be discussed. ORF 4 comprises 198 amino acids encoding a 25.6 kDa coat protein. The 16 amino acid sequence of the N-terminus of the viral coat protein was identical to that deduced from the ORF 4 nucleotide sequence (data not shown). Sequence similarity searches indicated that the RGMoV coat protein revealed a weak but significant similarity, 24 to 27% identity with that of SBMV, LTSV and RYMV, but only 15% identity with CfMV (Table 1).

In vitro translation of RGMoV RNA

In the wheat germ extract system, RGMoV RNA directs the synthesis of two products of 103 kDa and 68 kDa, but no other distinct product was detected. In contrast, the translational products synthesized *in vitro* with CfMV/JP RNA are four major proteins with sizes almost identical to those previously reported for CfMV/NO¹⁸ (Fig. 4). The translational activity of CfMV/JP RNA was low in our present system, as reported for other sobemoviruses¹⁶. RGMoV RNA is a poorer message in our wheat germ extract system.

The largest product of RGMoV RNA was 103 kDa and seems to be derived from the largest ORF 2 for the polypeptide. In the RGMoV RNA sequence, no ORF corresponds to the second largest product of 68 kDa. The putative replicase of CfMV is translated as part of a single polypeptide by -1 ribosomal frameshifting

Fig. 4. *In vitro* translation products made by RGMoV RNA and CfMV RNA. Lane 1, Heat-denatured, 3 μ g RGMoV RNA. Lane 2, Heat-denatured, 3 μ g RGMoV RNA and the labeled product was immunoprecipitated with antiserum to RGMoV and loaded; Lane 3, Heat-denatured, 3 μ g CfMV RNA. Positions of protein size markers are shown on the left.

between two overlapping ORFs having a coding capacity for 60.9 kDa and 56.3 kDa proteins^{7,18}). Translational frameshifts are known in coronavirus IBV²), polymerase genes of retroviruses^{5,6}) and plant viruses^{7,9}). As consensus signals for frameshifting, the heptanucleotide sequence (*e.g.*, UUUAAAC sequence) and the stem-loop structure immediately downstream have been proposed by Jacks *et al.*⁵). As found in CfMV, SBMV and RYMV⁷), identical signals are found in RGMoV RNA just preceding the initiation codon of the ORF 3 (Fig. 5). Tamm *et al.*¹⁸) proposed a possible mechanism that the 70 kDa *in vitro* translation product of SBMV and RYMV RNAs may represent the ORF 2-ORF 3 transframe fusion protein. Thus, the 68 kDa translational product of RGMoV RNA is probably derived from -1 ribosomal frameshifting (Fig. 3), not from proteolytic cleavage of the polyprotein²⁴). In this experiment, we tried to detect the RGMoV coat protein in the *in vitro* translation products by immunoprecipitation. However, we could not detect any signal for the coat protein. The coat protein of SBMV is translated only from a smaller, subgenomic RNA, which is detected in virus-infected tissues as well as virus particles¹⁴). As smaller RNAs were not detectable in our RGMoV RNA preparation, the amount of subgenomic RNA, if any, may have been insufficient for the detection of the *in vitro* translated coat protein.

Fig. 5. Consensus signals for frameshifting in RGMoV and sobemoviruses: heptanucleotide, UUUAAAC and a stem-looped structure. The number pointing to the first nucleotide in a slippery site indicates the actual site in the genome. The initiation codon of the RGMoV ORF 3 is boxed.

We conclude that RGMoV is a member of the genus *Sobemovirus* based on sequence similarities. The similarity level of nucleic acid (approximately 50% identity) and protein (Table 1) is low enough for virus species demarcation between any species of sobemoviruses, whereas the genome organization of RGMoV is closely related among sobemoviruses. Biological and serological properties of RGMoV are distinct from those of other characterized grass viruses²⁰⁾. Thus, RGMoV is a unique species of the genus *Sobemovirus*^{4,23)}. The polyprotein gene organization of RGMoV is the same as that of SBMV, RYMV and LTSV, but different from that of CfMV, for which a polyprotein is produced as a single fusion-protein by the frameshifting of two ORFs⁷⁾.

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