Ryegrass Mottle Virus, a New Virus from Lolium multiflorum in Japan

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Abstract

Ryegrass mottle virus (RMotV), a previously undescribed mechanically transmissible virus, was obtained from field infected Italian ryegrass (Lolium multiflorum) and cocksfoot (Dactylis glomerata) in Japan. The virus causes mottling and necrotic symptoms in leaves and readily infects wheat (Triticum aestivum), barley (Hordeum vulgare), oat (Avena sativa), Italian ryegrass and Italian millet (Setaria italica). Cocksfoot, chewing fescue (Festuca rubra) and perennial ryegrass (Lolium perenne) were less readily infected. The virus particle is isometric and ca. 28 nm in diameter. The particles have a sedimentation coefficient ($S_{20,w}$) of 108 S and a buoyant density of 1.366 g/ml in CsCl. They contain a single RNA component of mol. wt ca. $1.5 \times 10^6$, representing about 23% of the particle weight, a major protein of mol. wt 26,000 and possibly two minor proteins of 17,500 and 16,500. In double-diffusion tests, antiserum to RMotV reacted weakly with cocksfoot mottle virus and phleum mottle virus, apparently not to a common antigen. No relationship to RMotV was detected with antisera to cocksfoot mild mosaic, phleum mottle, cocksfoot mottle and cynosurus mottle viruses. RMotV is considered to be a newly discovered virus and a member of the phleum mottle virus group. Its present cryptogram is R/1: 1.5/23: S/S: S/*.

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Key Words: ryegrass mottle virus, properties, serology, grasses.

Introduction

Ryegrass mottle virus (RMotV), a virus with isometric particles ca. 28 nm in diameter, was found in Japan in Italian ryegrass (Lolium multiflorum) and cocksfoot (Dactylis glomerata) in a 5-year sward of Italian ryegrass, cocksfoot and white clover (Trifolium repens)\(^4\). Infected plants of Italian ryegrass were slightly stunted and leaves showed chlorotic mottling; leaves of infected cocksfoot plants had necrotic or chlorotic streaks or spots.

Of the five viruses with isometric particles which are known to infect grass species, four have a single RNA component: cocksfoot mottle virus (CfMV)\(^2,25,27\), cocksfoot mild mosaic virus (CMMV)\(^9,10,19\), phleum mottle virus (PMV)\(^4,5\) and
cynosurus mottle virus (CyMV)\(^5,16,17\), originally named lolium mottle virus\(^1\). The fifth virus, Weidelgrasmosaik virus (=ryegrass streak virus), which was later identified as a strain of brome mosaic virus\(^16,23\) has a multipartite genome.

This paper describes some biological, physical and chemical properties of RMotV together with evidence that it is a previously undescribed virus which can be placed in the phleum mottle virus group\(^9\).

**Materials and Methods**

**Virus source and inoculum.** Naturally infected plants of Italian ryegrass were removed from the field and kept in a greenhouse. RMotV was transferred to and maintained in wheat or barley seedlings by manual inoculation.

**Purification.** About 80 g of infected wheat or oat leaves were ground in 250 ml of 0.3 M borate buffer pH 6.0 containing 3 mM EDTA. The crude extract was clarified by treatment with 20% (v/v) of a mixture of n-butanol and chloroform (1:1) and the aqueous phase recovered by low speed centrifugation. The virus particles were sedimented by centrifugation for 2 hr at 123,000 \(\times g\) and the sediments resuspended in 0.03 M borate buffer pH 6.5 containing 1 mM EDTA. After centrifugation for 10 min at 5,500 \(\times g\), the supernatant fluid was centrifuged through 10-40% linear sucrose density gradient for 2 hr at 24,000 rev/min (RPS 25-2A rotor, Hitachi, Ltd., Japan). The virus containing band was removed with a syringe, diluted with the resuspending buffer and the virus particles sedimented by centrifugation. Final resuspension of virus particles was in 0.03 M borate buffer pH 6.5 containing 1 mM EDTA.

**Serology.** Antiserum to the virus was prepared by injecting a rabbit with a total of 20 mg purified preparations of virus particles by one intramuscular and three intravenous injections at 7 day intervals. Agar gel diffusion tests were carried out in 1% agar or 0.5% agarose containing 0.85% NaCl, 5 mM EDTA and 0.1% Na-azide. Antiserum to CfMV was prepared as described by Toriyama\(^26\). Other antisera used were supplied by the following: to PMV (Scottish isolate) by Dr. B.D. Harrison, Scottish Crop Research Institute, Dundee, Scotland; to CMMV and CyMV by Dr. P.L. Catherall, Welsh Plant Breeding Station, Aberystwyth, Dyfed, Wales.

**Electrophoresis of coat protein.** The coat protein of RMotV was analysed in SDS-10% polyacrylamide slab gels as described by Laemmli\(^12\). Molecular weight marker proteins were bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000) and cytochrome c (12,400).

**Preparation, electrophoresis and thermal denaturation of nucleic acids.** Nucleic acids of RMotV and CfMV were extracted and analysed as described previously\(^27\). After twice phenol extractions, the aqueous phase was still cloudy. Pretreatment of viral preparation with proteinase K (Kaken Chemicals, Tokyo, Japan) and addition of 10% m-cresol to the phenol mixture did not improve the clarity of extracts. When ethanol precipitated-nucleic acid was dissolved in 0.02 M Tris–HCl pH 7.5, some precipitation occured and it was removed by centrifugation. The precipitate was later confirmed to be viral nucleoprotein from the U. V. absorption curve and polyacrylamide gel electrophoretic analysis. RNA of rice dwarf virus\(^11\) was prepared by the same method. Yeast RNA was purchased from Sigma Chemical
Company (U.S.A.). The solvent for the RNAs used in the thermal denaturation experiment was 0.01×SSC (SSC: 0.15 M NaCl+0.015 M Na-citrate). The thermal denaturation profiles were obtained on a Gilford spectrophotometer 240 (Gilford Instrument Laboratories Inc., U.S.A.) equipped with constant temperature circulators (Haake Instruments, Inc., West Germany).

**Analytical centrifugation.** Virus preparations diluted to 0.6–3.0 mg/ml were examined in an analytical ultracentrifuge Hitachi Model 282 with 60 H rotor and Schlieren optics (Hitachi, Ltd., Japan).

**Buoyant densities of viruses.** Purified preparations of virus particles were centrifuged in CsCl (initial densities of CsCl, 1.4, 1.38 and 1.36 g/ml) at 106,000×g for 40–43 hr. Fractions of 0.1 ml were collected with an ISCO-fractionator (The Instrumentation Specialities Company, U.S.A.). The refractive index of each fraction was measured with an Abbe refractometer (Atago Co. Ltd., Tokyo, Japan). Centrifuge tubes containing CsCl and tubes containing fractions were covered with liquid paraffin to prevent evaporation.

**Results**

**Field symptoms and occurrence**

Naturally infected ryegrass plants were stunted and with chlorotic mottle in leaves (Fig. 1A, B). Chlorosis was sometimes accompanied by red to purple discoloration of the leaf tip. Infected cocksfoot plants showed brown to orange-discoloured necrotic streaks and spots in leaves. In tests on four cocksfoot plants with such symptoms, CfMV was not detected but RMotV was found in all (Fig. 3C, D). Ryegrass plants showing chlorotic mottle were wide spread in the field which was about 2 ha in size. By contrast the diseased cocksfoot plants occurred only sporadically found in the field. A precise assessment of the incidence of the mottling disease was not made. However, compared to the mosaic disease which is caused by a filamentous virus and which is wide spread in old ryegrass field in Japan \(^{13}\), the incidence of the mottling disease seemed to be limited.

**Host range and symptom in experimentally infected plants**

RMotV readily infected wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereale*), oat (*Avena sativa*) and Italian ryegrass (*Lolium multiflorum*) causing chlorotic stripe or mottling, sometimes accompanied by necrosis (Fig. 1C). Chlorosis and chlorotic or necrotic spots appeared in inoculated leaves of barley, Italian millet (*Setaria italica*) and sometimes in those of wheat. Systemic infection did not occur in Italian millet. Perennial ryegrass (*L. perenne*), cocksfoot (*Dactylis glomerata*) and chewing fescue (*Festuca rubra*) were infected by RMotV but with difficulty. Symptoms in perennial ryegrass were similar to those in Italian ryegrass. The following species were not infected: *Agrostis alba*, *A. teniins*, *Bromus inermis*, *Digitaria ascendentens*, *Echinochloa crus-galli*, *Eleusine indica*, *Festuca eratior*, *Oryza sativa*, *Phleum pratense*, *Sorghum bicolor*, *Sorghum spp.*, *Setaria viridis* and *Zea mays* (Table 1).
Fig. 1. A) Italian ryegrasses artificially inoculated with RMoV (left) compared with uninoculated (right). Inoculated plants show chlorotic motting and necrosis on the leaves. The growth was apparently retarded as ryegrasses naturally infected in field.

B) Chlorotic motting on the leaves of naturally infected Italian ryegrass (upper three leaves) and an uninfected leaf.

C) Chlorotic and necrotic symptoms on uninoculated barley leaves infected with RMoV.

Table 1. Host range of ryegrass mottle virus

<table>
<thead>
<tr>
<th>Species</th>
<th>Infectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Back-inoculation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Species</th>
<th>Infectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Back-inoculation&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Agrostis alba</td>
<td>-</td>
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<td>cv. Arborio J</td>
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<tr>
<td>A. tenness</td>
<td>-</td>
<td></td>
<td>cv. Mangetsu-mochi</td>
<td>-</td>
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<tr>
<td>Avena sativa</td>
<td>+</td>
<td></td>
<td>cv. Norin # 8</td>
<td>-</td>
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<tr>
<td>Bromus inermis</td>
<td>-</td>
<td></td>
<td>cv. Yamabiko</td>
<td>-</td>
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<tr>
<td>Dactylis glomerata</td>
<td>+ (n)</td>
<td></td>
<td>Pheum pratense</td>
<td>-</td>
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<tr>
<td>cv. Aonami</td>
<td>+(n)</td>
<td></td>
<td>Secale cereale</td>
<td>+</td>
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<tr>
<td>cv. Okamidori</td>
<td>+ (n)</td>
<td></td>
<td>Sorghum bicolor</td>
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<tr>
<td>Digitaria ascendens</td>
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<td>Sorghum spp.</td>
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<tr>
<td>Echinochloa crus-galli</td>
<td>-</td>
<td></td>
<td>cv. Green sorghum</td>
<td>-</td>
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<tr>
<td>Eleusine indica</td>
<td>-</td>
<td></td>
<td>cv. Sweet sorghum</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Festuca eriator</td>
<td>-</td>
<td></td>
<td>Setaria italica</td>
<td>+ (N)</td>
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<tr>
<td>F. rubra</td>
<td>-</td>
<td></td>
<td>S. viridis</td>
<td>-</td>
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<tr>
<td>Hordeum vulgare</td>
<td>+</td>
<td></td>
<td>Triticum aestivum</td>
<td>+ (N, n)</td>
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<tr>
<td>Lolium multiflorum</td>
<td>+ (N, n)</td>
<td></td>
<td>Zea mays</td>
<td>-</td>
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<tr>
<td>L. perenne</td>
<td>+</td>
<td></td>
<td>cv. Golden cross bantam</td>
<td>-</td>
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<tr>
<td>Oryza sativa</td>
<td>-</td>
<td></td>
<td>cv. Honey bantam</td>
<td>-</td>
<td></td>
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<tr>
<td>cv. Asahi</td>
<td>-</td>
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<sup>a</sup> -, no symptom; +, > 80% of inoculated plants infected; +, <10% of inoculated plants. N and n, conspicuous necrotic spots or streaks in inoculated (N) and uninoculated (n) leaves.

<sup>b</sup> Inoculated to wheat seedlings. - , no symptom.
Purification of virus particles

Examination of leaves of infected Italian ryegrasses and wheat plants by the leaf dip-method\(^6\) indicated that virus particles were present in high concentration. The isometric particles, stained with 2% phosphotungsteic acid pH 6.5 were ca. 28 nm in diameter (Fig. 2A). Preparation of virus particles centrifuged through sucrose density gradients produced one strongly light scattering band and a more faint zone below. The yield of purified virus by the method employed was 80-100 mg per 100g of fresh wheat leaves. Virus particles sedimented by high speed centrifugation were readily dissolved in the resuspending buffer by gentle shaking. \(A_{260}/A_{280}\) ratio of purified preparations was 1.62±0.01.

Stability in crude sap and in purified preparation

Sap from infected wheat leaves, extracted in 0.03 M borate buffer pH 6.5, was infective when diluted to \(10^{-5}\) but not \(10^{-6}\), after heating for 10 min at 85°C but not at 90°C and after storage at 5-15°C for 2 months. Purified preparations were infective after storage for one year at 4°C.

Serology

The titre of the RMotV antiserum was 1/1024 in precipitin tests. Antiserum to the virus reacted faintly with CfMV (Fig. 3A), but the reaction was distinct from the homologous reaction, because as shown in Fig. 3A, C, precipitation bands crossed. On the other hand, the antiserum to CfMV did not react with RMotV particles (Fig. 3B). RMotV antiserum reacted faintly with PMV forming one or two precipitation lines (Fig. 3E). Antisera to PMV (Scottish isolate), CMMV and CyMV did not react with the RMotV (Fig. 3F, G). The weak reactions of CfMV and PMV with RMotV antiserum seemed to be distinct each other, for precipitation lines did not coalesce (Fig. 3H).

Analytical centrifugation

Particles of the virus were found to have a sedimentation coefficient \((S_{20,w})\) of 108 S, when S values were measured at concentrations of 0.5, 0.8, 1.0, 1.2 and 3.0
mg per ml of virus solution and extrapolated to infinite dilution (Fig. 2B). The sedimentation coefficient was unaffected by the addition of 1 mM or 5 mM EDTA to the virus suspension (0.03 M borate buffer pH 6.5; virus concentration, 3.0 mg/ml).

**Buoyant densities**

When centrifuged to equilibrium in CsCl the virus particles formed a single sharp
band and the buoyant density was 1.366 g/ml. The buoyant density of CfMV determined under the same conditions was 1.378 g/ml. The percentage weight of RNA in the virus particle was about 23% as calculated from the formula of Sehgal et al.²⁴)

Proteins

The protein prepared from particles of RMotV contained a single polypeptide of mol. wt 26,000. This was smaller than that of CfMV, mol. wt 29,000 (Fig. 4a–f). When increased amounts of sample were applied to the gels, two minor bands were also obtained with RMotV but not with preparations made from CfMV particles. Mol. wt of these two minor bands was 17,500 and 16,500, respectively (Fig. 4g–i).

Nucleic acid

Phenol-extracted nucleic acid preparations showed a maximum absorbance at 256.5 nm and minimum absorbance at 228.5 nm. The ratios of A_{260}/A_{280} and A_{280}/A_{260} were 0.463 and 0.435, respectively. The nucleic acid was shown to be RNA from the positive orcinol colour reaction. Mol. wt of the virus RNA was approximately 1.5 × 10⁶. The RNA seemed to be degraded into smaller molecules when stored at −20°C. In freshly made RNA preparations of RMotV and CfMV, minor bands were not observed. When these preparations were coelectrophoresed, a single, slightly broad band was obtained.

Fig. 5 shows the U. V. absorption of RNAs in relation to temperature. Rice dwarf virus-RNA, which is a double-stranded RNA¹⁵), showed a steep rise around the melting temperature (80°C). The melting curves of native RNA of RMotV, yeast RNA (Fig. 5) and CfMV RNA (not shown in the figure) are characteristic of a single-stranded RNA with some secondary structure⁷): a temperature dependent increase in U. V. absorption was observed over a wide range.

Discussion

Paul et al.²²) placed 17 isolates of isometric grass viruses into several groups according to their serological interrelationship. RMotV has no common antigen with CfMV, PMV, CMMV and CyMV, and is also distinct from these viruses and other grass viruses so far reported in host range and symptomatology⁴,⁵,²⁸). Thus the virus from ryegrass in this study seemed to be a new virus and is named ryegrass mottle virus. Its present cryptogram is R/1: 1.5/23: S/S: S/*.
Hull\textsuperscript{8} classified small spherical viruses with a single RNA component into three groups according to the protein mol. wt., sedimentation coefficient, banding behavior in Cs\textsubscript{2}SO\textsubscript{4} and particle stability. Torrance and Harrison\textsuperscript{28} stated that particle stability of CMMV differs with the isolate and therefore is a less useful character than the others in grouping these viruses. The stability of RMotV has not been investigated in detail but the size of the coat protein suggests that RMotV can be assigned to the phleum mottle virus group\textsuperscript{8}. PMV and CMMV are serologically related and they, if not all isolates of these viruses, infect Setaria spp.\textsuperscript{5,21,28} As RMotV infects Setaria italica, infection on Setaria spp. might be a useful biological character for grouping of grass viruses into CMMV (PMV) or CfMV (CyMV) groups.

RMotV has two minor proteins as well as major protein of mol. wt 26,000. Paul \textit{et al.}\textsuperscript{22} also described minor protein components in Holcus transitory mottle virus and PMV. The origin and function of these minor protein components, however, is uncertain. Further investigations are required on the relationships between these minor components and also on the weak serological reaction shown by CfMV and PMV particles and antiserum to RMotV and other grass viruses\textsuperscript{8,20}.

Although lolium mottle virus (LMV) was originally isolated from \textit{Lolium perenne}\textsuperscript{1}, it did not infect \textit{Lolium} spp. in later studies and therefore was renamed CyMV\textsuperscript{5}. The mol. wt of CyMV protein, 30,500 is nearer to that of southern bean mosaic virus by Mohamed\textsuperscript{16} and he proposed that LMV as examined by Hull\textsuperscript{8} should be placed into the southern bean mosaic virus group and not in the phleum mottle virus group. However, the mol. wt of coat protein, 25,000, and buoyant density, 1.360 g/ml, of LMV found by Hull\textsuperscript{8} are nearer to those of RMotV, 26,000 and 1.366 g/ml, respectively. Therefore it would be of interest to examine, if possible, the isolates of LMV used in these different studies to determine whether they were indeed all isolates of the same virus\textsuperscript{1,5,8,16}.

\textbf{Acknowledgement}

The authors wish to express sincere appreciation to Drs. B.D. Harrison and W.P. Mowat, Scottish Crop Research Institute, Dundee, Scotland, for a gift of antiserum to phleum mottle virus (Scottish isolate) and for their kind help with the preparation of this manuscript and Dr. P.L. Catherall, Welsh Plant Breeding Station, Dyfed, Wales for a gift of antisera to cocksfoot mild mosaic virus and cynosurus mottle virus.

\textbf{Literature cited}

和文摘 要

イタリアンライグラス（Lolium multiflorum）の斑紋
萎縮病から分離された新ウイルス、ライグラス
モットルウイルス

島山光重・御子柴義郎・土居雅二

イタリアンライグラスで、やや萎縮し、葉に黄色の条斑紋を示す斑紋萎縮病から分離されたウイルスは、
汁液接種でイタリアンライグラス、コムギ、オオムギ、オオバク、ライムギ、アワに高率で感染し、オーチャードグラス、ペレニアルライグラス、チュービングフェスタにも低率であるが感染した。オオムギ、オーチャードグラス、アワなどでは死症状を伴うことが多い。本ウイルスの野外での発生については十分調査していないが、本病発生地では、イタリアンライグラスにかなりの発生がみられ、またオーチャードグラスでも発生が認められた。ウイルス粒子は直径約28 nmの球形で、粒子量約1.5×10⁶の一部をRNAを含み、蛋白の分子量は28,000 dである。核酸含量は粒子重の約23%、CsCl中の浮遊密度は1.366 g/ml、粒子の沈降定数は108 Sであった。本ウイルス抗血清はニックスフットモットルウイルス（CfMV）およびphleum mottle virus（PMV）と弱い反応を示したが、本ウイルスとの沈降帯は血清学的に明らかに異なる。またイネ科草類を宿主とし、類似の粒子性状をもつcocksfoot mild mosaic virus（CMV）およびryegrass mottle virus（PMV）の抗血清は本ウイルスと全く反応しなかった。本ウイルスは新ウイルスと考えられたので、ライグラスマットルウイルス（英名：ryegrass mottle virus）と命名した。外被蛋白の分子量、Setaria 属植物に対する寄生性や粒子の物理的性状から、CfMV グループよりは PMV グループ（Hull, 1977）に属すると思われる。クリプトグラムは、R/1：1.5/23：S/S：S/*と表わされる。