Two Purified RNAs of Soil-borne Wheat Mosaic Virus are Needed for Infection

By YUKIO SHIRAKO*† AND MYRON K. BRAKKE

Agricultural Research Service, U.S. Department of Agriculture and Department of Plant Pathology, Nebraska Agricultural Experiment Station, University of Nebraska, Lincoln, Nebraska 68583, U.S.A.

(Accepted 13 September 1983)

SUMMARY

RNAs of soil-borne wheat mosaic virus (SBWMV) from virions 281 nm, 138 nm and 92 nm long (designated here by relative lengths as 1-0L, 0-5L and 0-35L, respectively), were isolated and purified by three cycles of sucrose density gradient centrifugation. Infectivity assays with these RNAs proved the bipartite nature of SBWMV, the combination of 1-0L and either 0-5L or 0-35L RNAs being required for infection and for multiplication of progeny viruses. The 0-5L RNA underwent deletion mutation, producing smaller variants with various sizes, of which 0-4L and 0-35L RNAs were confirmed to be functional in combination with 1-0L RNA. The coat proteins of all isolates had mol. wt. of 19700. The mol. wt. of 1-0L, 0-5L, 0-4L and 0-35L RNAs, determined under denaturing conditions, were 2.28 × 10^6 (6500 bases), 1.23 × 10^6 (3500 bases), 0.97 × 10^6 (2800 bases) and 0.86 × 10^6 (2450 bases), respectively. A new virus group, furovirus (fungus-borne rod-shaped virus), is proposed for SBWMV.

INTRODUCTION

Soil-borne wheat mosaic virus (SBWMV) causes mosaic, stunting, and up to 50% loss of yield in winter wheat in the United States, Japan and Italy (Brakke, 1971; Palmer & Brakke, 1975; Campbell et al., 1975). Polymyxa graminis Led., a plasmodiophoraceous fungus, is the vector and is responsible for persistence of the virus in infested fields (Estes & Brakke, 1966; Rao & Brakke, 1969; Brakke et al., 1965).

SBWMV has hollow, stiff rod-shaped virions 20 nm in diameter with single-stranded RNA (Gumpf, 1971). All isolates have at least two components, the larger designated virion I (281 to 300 nm long) and the smaller, virion II (138 to 160 nm, or 92 to 110 nm long) (Gumpf, 1971; Brakke et al., 1965; Tsuchizaki et al., 1973; Brakke, 1977). Part of the variation in reported length is artefactual, and part is real. In this report, the virions will be designated by the ratio of their lengths to that of virion I, i.e. 281 to 300 nm, 138 to 160 nm and 92 to 110 nm long virions as 1-0L, 0-5L and 0-35L virions, respectively. This nomenclature has been adopted because it does not depend on function or relationships of the various components, it is easily expanded as virions with additional characteristic sizes are found, and it can be based on either electron microscopic determination of virion length or on estimates of RNA molecular weight.

Interpretation of previous research on function of components of SBWMV has been complicated by incomplete separation due to aggregation of the virions. Intact 0-35L or 0-5L virion IIs and RNA from 0-5L virions, fractionated by sucrose density gradient centrifugation, were non-infectious (Gumpf, 1971; Tsuchizaki et al., 1975). Preparations of virion I separated by sucrose density gradient centrifugation were infectious, but these results did not prove that virion I by itself was infectious, because 0-35L or 0-5L virions were detected in the preparations by electron microscopy (Gumpf, 1971; Tsuchizaki et al., 1975). Brakke (1977) found approximately half the 0-5L virion IIs had sedimented in a sucrose density gradient as dimers to
the same depth as virion I. Tsuchizaki et al. (1975) reported that mixing the virion I fraction from sucrose density gradients with the virion II fraction enhanced infectivity. In reassortment experiments with virion Is and IIs from different strains, they obtained some progeny with properties corresponding to those of the strain furnishing virion II. These results suggested that both virion I and virion II are needed for infectivity and both are genetically active, but that conclusion merits confirmation because of the incomplete separation of the virions by sucrose density gradient centrifugation.

The relationship between 0.35L and 0.5L virion IIs has also been uncertain. Virions purified from naturally infected wheat in the spring always have 1.0L virions and 0.5L virions, and in addition sometimes have major amounts of virions with a modal length between 0.35L and 0.5L, as well as the usual minor amounts of rods of other lengths as is common to all rod-shaped plant viruses (Brakke, 1977). Successive manual transmission of a culture with virions of three lengths (0.35L, 0.5L and 1.0L) resulted in separation of two types of cultures, one with 0.5L and 1.0L virions, and the other with 0.35L and 1.0L virions (Brakke, 1977). There are two explanations for the relationship between 0.35L and 0.5L components. Two strains (one being 0.5L plus 1.0L, the other 0.35L plus 1.0L) may coexist in the field, or the 0.35L, and others shorter than 0.5L, may arise by deletion mutation from the 0.5L component. Results presented here suggest that the latter hypothesis is correct.

**METHODS**

**Virus sources and maintenance.** Two isolates of SBWMV were used. One (wild-type; WT) was obtained in the spring from naturally infected wheat near Lincoln, Nebraska. Another (Lab 1) originated from WT by successive manual transfers at bimonthly intervals for 2 years. For manual transfers, the infected leaves were ground in 0.1 M-K₂HPO₄ in a mortar and pestle and the extract was inoculated to wheat plants (Triticum aestivum L. cv. 'Michigan Amber') at the two-leaf stage with Celite as an abrasive. The inoculated plants were kept in the dark for 5 days to enhance virus spread in the plant and subsequently were grown in environmental chambers at 17 °C with 20000 lux of cool white fluorescent light (Rao & Brakke, 1970). Infected leaves were stored at -20 °C until purification.

**Virus purification.** One-hundred g of infected leaves was ground in 150 ml of 0.5 M-sodium borate pH 9.0, 1 mM- Na₂EDTA in a Waring blender. The extract was squeezed through a double layer of cheesecloth and centrifuged at 10000 rev/min for 10 min in a Beckman JA-20 rotor. After addition of Triton X-100 to 2%, the supernatant was layered onto a pad of 6 ml of 20% sucrose in grinding buffer and centrifuged at 28000 rev/min for 2 h at 4 °C in a Beckman type 30 rotor. The resulting pellet was resuspended in 0.05 M-sodium borate pH 8.0, 1 mM-Na₂EDTA and subjected to the second cycle of centrifugal fractionation. The final pellet was resuspended in 2 ml of distilled water. This viral suspension was frozen at -20 °C overnight, thawed without shaking, and centrifuged at 6000 rev/min for 5 min in a Beckman JA-20 rotor. The supernatant was stored at -20 °C until use. The approximate virus yield was 2 mg per 100 g of infected leaves.

**Extraction of viral RNA.** Purified virus was dissociated by storage for 16 h at 4 °C in 0.1 M-ammonium carbonate, 1% SDS, 1 mM-Na₂EDTA, pH 9.0, containing 200 µg bentonite/ml (Brakke & Van Pelt, 1970). The released RNA was separated from the residual proteins by centrifugation through a 10 to 40%, linear sucrose density gradient prepared in 0.1 M-ammonium carbonate, 0.3 M-ammonium chloride, pH 9.0, at 38000 rev/min for 7 h at 14 °C in a Beckman SW41 rotor. The gradient was fractionated with an ISCO Model 183 density gradient fractionator and zones of each RNA were collected with the aid of an ISCO UA-5 absorbance monitor. Each isolated RNA was subjected to the second cycle of differential centrifugation. The final pellet was resuspended in 2 ml of distilled water. This viral suspension was frozen at -20 °C overnight, thawed without shaking, and centrifuged at 6000 rev/min for 5 min in a Beckman JA-20 rotor. The supernatant was stored at -20 °C until use. The approximate virus yield was 2 mg per 100 g of infected leaves.

**Virus gel electrophoresis.** Virus was disrupted by incubation for 5 min at 60 °C in 2% SDS, 1% 2-mercaptoethanol in E buffer (10 mM-NaH₂PO₄, 20 mM-NaHPO₄, 1 mM-Na₂EDTA, pH 7.2) containing 200 µg bentonite/ml. Each virus suspension was rubbed singly and in combination with others onto wheat plants at the two-leaf stage with Celite as an abrasive. Inoculated plants were kept in the dark for 5 days and grown at 17 °C subsequently. The number of plants with symptoms were counted 6 weeks after inoculation.
**RNAs of soil-borne wheat mosaic virus**

![Sedimentation profiles of SBWMV-RNAs](image)

Fig. 1. Sedimentation profiles of SBWMV-RNAs in 10 to 40% linear sucrose density gradients. (a) Viral RNAs from WT; (b) 0.5L RNA from a slower-sedimenting zone of (a); (c) 1.0L RNA from a faster-sedimenting zone of (a); (d) viral RNAs from Lab 1; (e) 0.35L RNA from a slower-sedimenting zone of (d); (f) 1.0L RNA from a faster-sedimenting zone of (d); arrows indicate the positions of 1.0L, 0.5L and 0.35L RNAs from the right.

**RESULTS**

*Extraction and purification of viral RNAs*

The two species of RNAs were separated from each isolate by sucrose density gradient centrifugation after high pH, SDS disruption (Fig. 1). 0.5L and 1.0L RNAs were isolated from WT (Fig. 1a), whereas 0.35L and 1.0L RNAs were isolated from Lab 1 (Fig. 1d). Each RNA species was purified further by another two cycles of sucrose density gradient centrifugation. Judged by the ultraviolet absorption profile, each RNA was free from other RNAs (Fig. 1b,c,e,f). The ratio of the amount of 0.35L or 0.5L RNA to that of 1.0L RNA was always higher than 10:1, indicating that the number of 1.0L virions was always less than one-twentieth of that of 0.35L or 0.5L virions. This component ratio confirmed previous ratios obtained by counting virus particles observed in the electron microscope (Tsuchizaki, et al., 1973; Brakke, 1977).
Table 1. Infectivity determined by inoculation of single and mixed RNAs purified by three cycles of sucrose density gradient centrifugation

<table>
<thead>
<tr>
<th>RNA component(s)</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35L (Lab 1) + buffer*</td>
<td>0/27† (13)‡</td>
<td>0/51 (21)</td>
</tr>
<tr>
<td>0.5L (WT) + buffer</td>
<td>0/25 (7)</td>
<td>0/52 (21)</td>
</tr>
<tr>
<td>1.0L (Lab 1) + buffer</td>
<td>0/32 (0.4)</td>
<td>1/54 (2.2)</td>
</tr>
<tr>
<td>1.0L (WT) + buffer</td>
<td>0/24 (1.1)</td>
<td>0/46 (1.8)</td>
</tr>
<tr>
<td>0.35L (Lab 1) + 1.0L (Lab 1)</td>
<td>13/54 (21 + 2.2)</td>
<td></td>
</tr>
<tr>
<td>0.35L (Lab 1) + 1.0L (WT)</td>
<td>8/68 (13 + 1.1)</td>
<td>9/57 (21 + 2.2)</td>
</tr>
<tr>
<td>0.5L (WT) + 1.0L (Lab 1)</td>
<td>4/99 (7 + 0.4)</td>
<td>9/59 (22 + 2.2)</td>
</tr>
<tr>
<td>0.5L (WT) + 1.0L (WT)</td>
<td>13/54 (21 + 2.2)</td>
<td></td>
</tr>
<tr>
<td>Buffer only</td>
<td>0/54 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Buffer consisted of 0.05 M-glycine, 0.05 M-K₂HPO₄, pH 9.2, containing 200 µg bentonite/ml.
† The number of plants showing mosaic leaf symptoms in the numerator and the number of plants inoculated in the denominator.
‡ Concentration of RNA in the inoculum, in µg/ml.

Infectivity assay

Infectivity assays were performed by the inoculation of isolated RNAs singly and in combinations. Infectivity of RNA was always low, i.e. at the highest 24% of the inoculated plants were infected, but the results clearly indicated an essential role for each RNA (Table 1). Neither 0.35L nor 0.5L RNA was infectious by itself, even if the concentration in the inoculum was relatively high. The 1.0L RNA had only a trace of infectivity, one of 54 inoculated plants developing symptoms in Expt. 2. Analysis by gel electrophoresis of extracted RNA showed this particular plant to be infected with 0.35L and 1.0L virions, indicating that the infection was probably due to the contamination by 0.35L RNA in the 1.0L RNA preparation. On the other hand, inoculation with mixtures of 0.35L and 1.0L RNAs and of 0.5L and 1.0L RNAs infected in all homologous and heterologous combinations. Infection by reassorted heterologous combination of RNAs indicates that 1.0L RNAs from both WT and Lab 1 isolates were compatible with both 0.35L and 0.5L RNAs. Therefore, there seems to be no specific difference between the two 1.0L virions in terms of their role in infectivity and multiplication with 0.35L or 0.5L virions.

Progeny viruses from reassorted RNA inoculation

Plants infected with the Lab 1 isolate had a severe yellow mosaic and were more stunted than those infected with WT isolate, which caused a green mosaic (Fig. 2). Among assay plants, those inoculated with 0.35L and 1.0L RNAs had a severe yellow mosaic and stunting. The virus from these plants contained only 0.35L and 1.0L RNAs regardless of the source of 1.0L RNA (Fig. 3). On the other hand, the individual assay plants inoculated with 0.5L and 1.0L RNAs developed different symptoms ranging from a mild mosaic to stunting accompanied by a severe mosaic. Progeny viral RNAs from combined assay plants inoculated with 0.5L RNA (WT) and 1.0L RNA (either WT or Lab 1) had three RNA peaks after density gradient centrifugation, one RNA smaller than 0.5L as well as 0.5L and 1.0L RNAs (data not shown). To examine the possible causes of the differences in symptoms and to confirm the unexpected density gradient centrifugation results, viruses were purified from the individual plants and their RNA compositions were analysed (Fig. 4). The virus from individual plants showed different patterns of RNAs. In some cases, RNAs smaller than 0.5L were detected along with 0.5L and 1.0L RNAs (Fig. 4, C, F, H, J, K, L, M, N, O and S). In other cases, there was no 0.5L RNA, but only smaller RNAs (Fig. 4, D, E, G, I, P, Q and R). There was an apparent positive correlation between the severity of symptoms and the amount of virion II RNA smaller than 0.5L.

Evidence of deletion mutation

In the particular case of plant P in Fig. 4, the virions consisted of 1.0L and an intermediate size between 0.5L and 0.35L, designated as 0.4L here. To examine the function and the progeny
RNAs of soil-borne wheat mosaic virus

Fig. 2

Fig. 2. Symptoms of SBWMV-infected wheat plants (*Triticum aestivum* L. cv. 'Michigan Amber'). Left (A); infected with Lab 1. Right (B); infected with WT.

Fig. 3

Fig. 3. Polyacrylamide–agarose composite gel pattern of progeny viral RNAs after the inoculation with reassorted 0.35L and 1.0L RNAs. RNAs from WT (a) and Lab 1 (b) were inoculated after reassortment; (c) progeny viral RNAs after the inoculation with 0.35L and 1.0L RNAs both from Lab 1; (d) progeny viral RNAs after the inoculation with combination of Lab 1 0.35L RNA and WT 1.0L RNA; (s) mol. wt. standards: TMV-RNA (2.19 × 10⁶) and *E. coli* ribosomal RNAs (1.01 × 10⁶ and 0.53 × 10⁶).

of this isolate (Lab 2), an extract of plant P was inoculated manually to wheat plants, which later developed symptoms. The progeny virus had only 0.4L and 1.0L RNAs 2 months after inoculation. These results indicate the genetic function and the structural stability of 0.4L RNA. However, further analysis of the progeny virus purified from infected plants after another several months showed the occurrence of 0.35L RNA in addition to 0.4L and 1.0L RNAs in one of six plants (data not shown).

**Molecular weight of RNAs**

The RNA molecular weights were determined after formaldehyde denaturation (Fig. 5). A preliminary investigation revealed that a plot of logarithm of molecular weight against distance of migration after formaldehyde denaturation of *E. coli* ribosomal RNAs and TMV RNA was linear when 1.7% agarose was used, but curved at higher or lower concentrations of agarose. Similar results were obtained after denaturation with 1.0 M-glyoxal as recommended by McMaster & Carmichael (1977). The molecular weights of 1.0L, 0.5L, 0.4L and 0.35L RNAs were 2.28 × 10⁶ (6500 bases), 1.23 × 10⁶ (3500 bases), 0.97 × 10⁶ (2800 bases) and 0.86 × 10⁶ (2450 bases), respectively. There was no significant difference in the size among the 1.0L RNAs from WT, Lab 1 and Lab 2 isolates.

**Molecular weight of coat protein**

The average molecular weight of the coat protein determined by electrophoresis in 10.0, 12.5 and 15.0% polyacrylamide gel, with or without 3-mercaptopropionic acid, was 19700. There was no difference in the molecular weight among the proteins from WT, Lab 1 and Lab 2 isolates (data not shown).
Fig. 4. Polyacrylamide-agarose composite gel pattern of progeny viral RNAs after the inoculation with reassorted 0.5L and 1.0L RNAs. (A) and (B): same as lanes (a) and (b) described in Fig. 3; (C to K) progeny viral RNAs after the inoculation with a combination of WT 0.5L RNA and Lab 1 1.0L RNA; (L to S) progeny viral RNAs after the inoculation with 0.5L and 1.0L RNAs both from WT; (s) mol. wt. standards (see Fig. 3).

DISCUSSION

Earlier investigators of SBWMV genetics separated virions I and II by sucrose density gradient centrifugation, a procedure satisfactory for virion II, but not for virion I which is usually contaminated by aggregated virion II (Brakke, 1977). The aggregation problem should be less with purified RNA than with SBWMV virions. This expectation was borne out by our results. Preparations of virion II RNA (0.35L and 0.5L RNAs) were non-infectious and those of virion I RNA (1.0L RNA) had only a trace of infectivity. A mixture was infectious, showing that both components were functional. The progeny viral RNAs after the inoculation with 0.35L and 1.0L RNAs were 0.35L and 1.0L RNAs, regardless of the source of RNA I, showing that RNA II is genetic. Plants inoculated with 0.5L and 1.0L RNAs had progeny virus with 0.5L and 1.0L RNAs, and also shorter RNAs. These shorter RNAs did not result from simple contamination because in that case only 0.35L RNA would have appeared in these plants. In fact, a variety of RNAs between 0.35L and 0.5L were found, and the pattern of RNA size distribution was different for each individual plant. At least one of the new RNAs, 0.4L RNA from plant P (Fig. 4), was shown to be functional. Neither 0.5L nor 0.35L RNA was detected in virus from this plant. Nevertheless, the virus was infectious and progeny virus had 0.4L and 1.0L RNAs.
RNAs of soil-borne wheat mosaic virus

RNAs, with one exception. The exception was one plant in which 0.35L appeared in addition to 0.4L and 1.0L after a period of several months. From these results, we tentatively conclude that the RNAs shorter than 0.5L in plants inoculated with 0.5L and 1.0L RNAs arose by deletion mutation of 0.5L RNA.

The nature of deletion mutation explains the occurrence of variants of short rods of SBWMV reported before (Tsuchizaki et al., 1973; Brakke, 1977; Hibino et al., 1981). We have also found spontaneously occurring deletion mutations of 0.5L RNA both in plants mechanically inoculated with the WT isolate and in the naturally infected plants collected from the infested fields in late autumn and continuously grown in a growth chamber at 17 °C for several months (Y. Shirako & M. K. Brakke, unpublished results). Therefore, deletion mutation seems to be a common characteristic of SBWMV, regardless of the isolates and the modes of infection.

The mechanisms and the molecular basis of deletion have not been determined. Deletion might occur by excision of bases from either end or by deletion of internal portions of RNA followed by splicing of pieces. In either case, the location of the deletion must be somewhere outside the coat protein gene, which is located in RNA II (Tsuchizaki et al., 1975; Hsu & Brakke, 1983). To obtain 0.4L RNA from WT 0.5L RNA, approximately 700 bases would have to be deleted, and to obtain 0.35L RNA, approximately 1050 bases. Genetic information coded on the 0.35L RNA (2450 bases) may be the minimum requirement for infection and multiplication in combination with 1.0L RNA (6500 bases). Further deletion from 0.35L RNA is probably lethal for the virus. Other examples of deletion mutation in plant viruses have been reported with cauliflower mosaic virus (Hull & Howell, 1978; Howarth et al., 1981), pea enation mosaic virus (Adam et al., 1979), and wound tumour virus (Reddy & Black, 1974). All of them lose insect-transmissibility as the result of deletion of a segment of the viral genome. From this viewpoint, we are investigating the fungus-transmissibility of deletion-mutated SBWMV.

From a taxonomical point of view, the bipartite nature of SBWMV, proved directly in this study, necessitates establishing a distinctive virus group. So far, SBWMV has been considered
Table 2. Comparisons of major properties among soil-borne wheat mosaic, tobacco mosaic, beet necrotic yellow vein, and potato mop-top viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>SBWMV</th>
<th>TMV</th>
<th>BNYVV</th>
<th>PMTV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle length, nm</td>
<td>110-160 and 300*</td>
<td>300‡</td>
<td>65-105, 270 and 3901</td>
<td>125 and 290**</td>
</tr>
<tr>
<td>Particle diameter, nm</td>
<td>20*</td>
<td>18‡</td>
<td>201</td>
<td>18-20‡†</td>
</tr>
<tr>
<td>Coat protein mol. wt. × 10⁻³</td>
<td>19-7†</td>
<td>17-5‡</td>
<td>21-0¶</td>
<td>19-8‡‡</td>
</tr>
<tr>
<td>RNA mol. wt. × 10⁻⁶</td>
<td>0-86-1-23 and 2-28†</td>
<td>2-19§</td>
<td>0-6, 0-7, 1-8 and 2-3¶</td>
<td>ND§§</td>
</tr>
<tr>
<td>Vector</td>
<td>Polymyxa graminis*</td>
<td>Unknown‡</td>
<td>Polymyxa betae</td>
<td>Spongospora subterranea††</td>
</tr>
<tr>
<td>Host range</td>
<td>Narrow*</td>
<td>Wide‡</td>
<td>Narrow‡</td>
<td>Narrow††</td>
</tr>
<tr>
<td>Genomic composition</td>
<td>Bipartite†</td>
<td>Monopartite‡</td>
<td>(Multipartite)¶</td>
<td>(Bipartite)¶¶</td>
</tr>
</tbody>
</table>

* Brakke (1971); † this study; ‡ Zaitlin & Israel (1975); § Casper (1963); † Tamada (1975); ¶ Putz (1977); ** Roberts & Harrison (1979); †† Harrison (1974); ‡‡ Randles et al. (1976); §§ ND, not determined.

to be a tentative member of the tobamovirus group (Gibbs, 1977), because of the morphological similarity and the slight serological relationship with TMV (Powell, 1976). However, SBWMV is fundamentally different from TMV, which is a classical monopartite virus. In addition, the molecular weight of the coat protein of SBWMV is significantly higher, by about 2000, than that of TMV, and RNA I of SBWMV is slightly larger than TMV RNA. We propose a new virus group, furovirus (fungus-borne rod-shaped virus) with SBWMV as the type virus. Major criteria of this new virus group should include a rigid, hollow rod-shaped virion, plasmodiophoraceous fungus-transmissibility, and divided genome composition. Together with SBWMV, other tentative members of the new virus group are beet necrotic yellow vein virus (Tamada, 1975), potato mop-top virus (Roberts & Harrison, 1979), <i>Hypochoeris</i> mosaic virus (Brunt & Stace-Smith, 1978; Greber & Finlay, 1981), <i>Nicotiana velutina</i> mosaic virus (Randles et al., 1976) and peanut clump virus (Thouvenel et al., 1976). Table 2 shows the comparisons of several major properties among SBWMV, TMV, BNYVV and PMTV.

Cooperative investigations by Agricultural Research Service, U.S. Department of Agriculture and Plant Pathology Department, Nebraska Agricultural Experiment Station, Lincoln, Nebraska 68583. Published with the approval of the Director as Paper No. 7017, Journal Series, Nebraska Agricultural Experiment Station.

REFERENCES


(Received 2 June 1983)