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Sequence Relationships among Soil-borne Wheat Mosaic Virus RNA Species and Terminal Structures of RNA II

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SUMMARY

Gel-separated RNAs of the wild-type (WT), Lab 1 and Lab 2 isolates of soil-borne wheat mosaic virus (SBWMV) were transferred to nitrocellulose filters and hybridized with specific cDNA. RNA I, but not RNA II, from all three SBWMV isolates hybridized to cDNA copied from SBWMV WT RNA I. RNA II from all three SBWMV isolates hybridized to cDNA copied from SBWMV WT RNA II, confirming the hypothesis that the Lab 1 and Lab 2 isolates arose by deletion of part of RNA II of WT virions. RNA II from SBWMV WT (0·5L RNA) or from SBWMV Lab 1 (0.35L RNA) did not bind to oligo(dT)–cellulose in high-salt buffer, or stimulate cDNA synthesis when primed with oligo(dT), suggesting that it has no 3′-poly(A) or oligo(A) sequences. No genome-linked protein was detected at the 5′ ends of either RNA II by iodination, nor did the cap analogue 7-methylguanosine phosphate have an effect on their translation *in vitro*, indicating no cap structure at their 5′ ends. RNA II was 5′ end-labelled with [^{32}P]ATP and T4 polynucleotide kinase after dephosphorylation with bacterial alkaline phosphatase, but all four bases were labelled, indicating heterogeneity at the 5′ end.

Soil-borne wheat mosaic virus (SBWMV) has a bipartite RNA genome separately encapsidated in rod-shaped virions (Shirako & Brakke, 1984*a*). Field plants usually have virions of two lengths, 281 and 138 nm, with RNA I of 2·28 × 10^6 mol. wt. and RNA II of 1·23 × 10^6 mol. wt. (WT isolate) (Brakke, 1977; Shirako & Brakke, 1984*a*). However, plants from some fields contain a variety of rods shorter than 138 nm. These plants were originally assumed to be infected by a mixture of strains. An isolate containing virions 281 and 92 nm long, called Lab 1, was isolated from one such mixture by repeated manual transmission. Results of reassortment experiments with purified RNA from Lab 1 and WT isolates confirmed that both RNA I and II were genetically functional (Shirako & Brakke, 1984*a*) as reported earlier by Tsuchizaki *et al.* (1975). However, Shirako & Brakke (1984*a*) also found that virions shorter than 138 nm frequently predominated after 4 to 5 months in plants originally inoculated with the WT isolate either manually or by the vector, *Polymyxa graminis* Led. (Shirako & Brakke, 1984*b*). The lengths of the predominant rods varied from plant to plant. Because of the variety of their lengths, the virions were designated by the ratio of their length to that of component I, which is the same as the ratio of the molecular weights of the RNAs. Component II of WT is 0·5L and of Lab 1 isolate is 0·35L. Another one of these short rods, 0·4L, also produced an infection when inoculated with RNA I and the combination was termed Lab 2 isolate (Shirako & Brakke, 1984*a*).

Shirako & Brakke (1984*a*) concluded that components smaller than 0·5L were deletion mutants of 0·5L RNA. We have studied properties of RNA II of WT, Lab 1 and Lab 2 isolates to obtain evidence concerning this deletion hypothesis and to identify the probable site of deletion. The properties of RNA I, which is only 5% of the total RNA by weight, were studied to a lesser extent.

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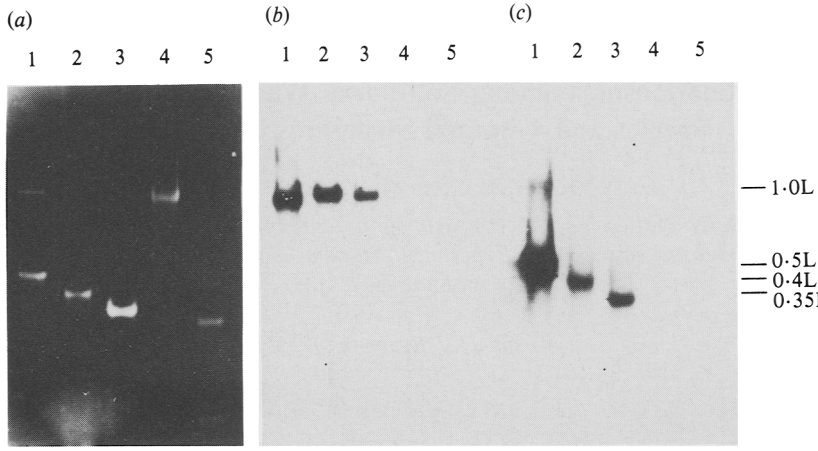

Fig. 1. Sequence relationships among RNAs of three isolates of SBWMV. One part of the gel was stained with ethidium bromide (*a*). The other parts were Northern blotted with ^{32}P-labelled WT 1·0L cDNA (*b*) or WT 0·5L cDNA (*c*). One μg of unfractionated RNA from WT (lane 1), Lab 2 (lane 2) and Lab 1 (lane 3) isolates of SBWMV and from tobacco mosaic virus (lane 4) and brome mosaic virus (lane 5) were denatured by formaldehyde and electrophoresed in an agarose gel in triplicate. The position of SBWMV RNAs are indicated on the right.

Northern blot hybridizations with randomly primed cDNA were done to find if 0·5L, 0·4L and 0·35L RNA had homologous sequences. WT, Lab 1 and Lab 2 SBWMV were purified from infected wheat plants (*Triticum aestivum* L., cv. Michigan Amber) grown in environmental chambers at 18 °C by differential and sucrose gradient centrifugation after treatment of sap with calcium phosphate and Triton X-100 (Y. H. Hsu & M. K. Brakke, unpublished). RNA was prepared from purified virions by disruption in SDS, phenol–chloroform extraction, and two cycles of sucrose density gradient centrifugation. RNA for cDNA synthesis was further purified by agarose gel electrophoresis in 5 mM-methylmercuric hydroxide (Bailey & Davidson, 1976; Weislander, 1979). The reverse transcription reaction was modified from that of Rosen & Barker (1976). Each 50μl of cDNA reaction mixture contained 50 mM-Tris–HCl (pH 8·3 at 42 °C), 140 mM-KCl, 10 mM-$MgCl_2$, 10 mM-dithiothreitol, 2 mg/ml actinomycin D, 100 μM of each deoxyribonucleoside triphosphate, 50 μCi [^{32}P]dCTP (New England Nuclear; 670 Ci/mmol), 5 μg RNA template, 5 μg primer, and 20 units of avian myeloblastosis virus reverse transcriptase (purchased from Dr J. Beard, Life Sciences, St. Petersburg, Fla., U.S.A.). A random primer was made by partial DNase hydrolysis of calf thymus DNA (Taylor *et al.*, 1976). The reaction proceeded for 1 h at 42 °C and was stopped by addition of 2 μl of 0·5 M-EDTA, 25 μl of 150 mM-NaOH and incubation for 1 h at 65 °C. The mixture was neutralized by adding 25 μl of 1 M-Tris–HCl pH 8·0, 25 μl 1 M-HCl, and was then extracted with phenol–chloroform. The cDNA was separated from unincorporated dNTP and the products of alkaline hydrolysis of the template by Sephadex G-50 chromatography.

For Northern blots, RNA was denatured in 6% formaldehyde (Boedtker, 1971), resolved by electrophoresis on a vertical 1·5% agarose gel, and transferred to nitrocellulose (Thomas, 1980). Prehybridization, hybridization with cDNA (20000 c.p.m./ml) and washing were as described by Gustafson *et al.* (1982). Hybridization was detected by autoradiography at −70 °C (Fig. 1). The cDNA from WT RNA I (i.e. 1·0L RNA) hybridized with 1·0L RNA from WT, Lab 1 and Lab 2 isolates but not with 0·5L, 0·4L or 0·35L RNA. The slight difference in migration rate of RNA I from different strains seen in Fig. 1 was due to deformation of the vertical agarose gel during preparation of the sample wells. The cDNA from 0·5L RNA of WT hybridized strongly with 0·5L, 0·4L and 0·35L RNA, showing the presence of common sequences among these three RNAs and supporting the conclusion that 0·4L and 0·35L RNAs are deletion mutants of 0·5L RNA. The weak hybridization between the cDNA to 0·5L RNA and 1·0L RNA could be the

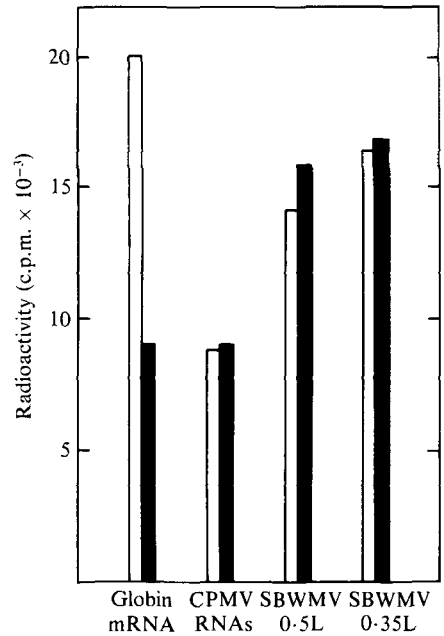

Fig. 2. Effects of cap analogue (m^7Gp) on translation. RNAs were translated in rabbit reticulocyte lysates either containing m^7Gp (■) or not (□) for 1 h. The incorporation of [^{35}S]methionine is the amount of radioactivity insoluble in 5% TCA. CPMV, Cowpea mosaic virus.

result of short common sequences, or of some type of artefact such as the presence of degradation products of 1·0L RNA in the 0·5L RNA template, or of electrophoretic artefacts (Palukaitis *et al.*, 1983). Probing with cloned cDNA or sequencing the RNA may be needed to resolve this problem.

Neither SBWMV 0·5L nor 1·0L cDNA hybridized with RNA from either brome mosaic virus or tobacco mosaic virus (Fig. 1). This is further evidence that SBWMV and tobacco mosaic virus are not closely related.

To find if the mutant RNA was formed by deletion of a portion near the 3′ end of 0·5L RNA, we attempted to make a cDNA of the 3′ end of 0·5L and 0·35L RNA by reverse transcription with an oligo$(dT)_{12-18}$ primer (Pharmacia P-L Biochemicals). Such attempts were unsuccessful, and suggested that SBWMV RNA II was not polyadenylated. This conclusion was confirmed by passing 50 μg of 0·5L RNA and of 0·35L RNA through a 2 ml oligo(dT)–cellulose (Type III, Collaborative Research, Lexington, Mass., U.S.A.) column in a high-salt buffer (20 mM-Tris–HCl pH 7·5, 0·5 M-NaCl, 1 mM-EDTA, 0·1% SDS). The column was washed with 8 ml of high-salt buffer, which removed 94% of 0·5L RNA and 91% of 0·35L RNA. A subsequent wash with low-salt (10 mM-Tris–HCl pH 7·5, 1 mM-EDTA, 0·05% SDS) buffer removed no detectable RNA. In a control experiment, 82% of cowpea mosaic virus RNA was recovered in the low-salt elution buffer, as expected because of its poly(A) tracts (El Manna & Bruening, 1973). Attempts to polyadenylate SBWMV 0·5L and 0·35L RNA with *Escherichia coli* poly(A) polymerase (Bethesda Research Laboratories) (Gilvarg *et al.*, 1975) were unsuccessful as judged by the failure to incorporate label from [^{3}H]ATP into material insoluble in TCA, and by the failure of the treated RNA either to serve as a substrate for reverse transcriptase with oligo(dT) as a primer, or to bind to an oligo(dT)–cellulose column.

The possibility that a genome-linked protein is at the 5′ end of SBWMV 0·5L and 0·35L RNAs was investigated by iodination. RNA (100 μg) was iodinated with 100 μCi carrier-free $Na^{125}I$ (Amersham) using iodogen (Pierce Chemical Co., Rockford, Ill., U.S.A.) as described by Fraker & Speck (1978) and separated from iodide by dialysis (against 10 mM-Tris–HCl pH 7·4, 1 mM-EDTA, 0·05% SDS) and ethanol precipitation. Iodinated RNA was mixed with 1 μg/ml bovine

serum albumin and digested with 1 μg/μl RNase A and 0·2 μg/μl RNase T1 in 10 mM-Tris–HCl pH 7·4, 1 mM-EDTA. After incubation at 37 °C for 4 h, the mixture was extracted with phenol and the protein in the phenol fraction was recovered as a precipitate by the addition of 10 vol. methanol and dissolved in electrophoresis sample buffer (Lane, 1978). Electrophoresis in an 8 to 25% polyacrylamide gradient gel containing SDS and with a discontinuous buffer system (Laemmli, 1970) followed by autoradiography revealed no labelled protein. Control experiments with cowpea mosaic virus RNA revealed a prominent protein at the expected position (Daubert *et al.*, 1978). We conclude that SBWMV 0·5L and 0·35L RNAs do not have a tyrosine-containing genome-linked protein.

To test for the possibility of a 7-methylguanosine cap at the 5′ end, 0·5L and 0·35L RNAs were translated in a rabbit reticulocyte lysate (Bethesda Research Laboratories) with or without the addition of 250 μM-m^7Gp. The standard 15 μl reaction mixture contained 1 μCi [^{35}S]methionine and either 0·15 μg of rabbit globin mRNA or 0·5 μg of virus RNA. The K^+ concentration was 135 mM for globin mRNA and 125 mM for virus RNA (Y. H. Hsu & M. K. Brakke, unpublished method). After 1 h at 30 °C translation was measured by the amount of radioactivity precipitated by 5% TCA. The presence of cap analogue had no effect on the translation of either 0·35L or 0·5L RNA of SBWMV, but reduced that of globin mRNA by 45% (Fig. 2).

These results indicated that 0·5L and 0·35L SBWMV RNAs had neither a 7-methylguanosine cap nor a genome-linked protein at the 5′ end. To find if they could be labelled at the 5′ end, 0·5L and 0·35L RNAs were dephosphorylated with bacterial alkaline phosphatase and treated with [γ-^{32}P]ATP and T4 polynucleotide kinase (Chaconas & Van de Sande, 1980). The labelled product migrated normally in denaturing gel electrophoresis with 5 mM-methylmercuric hydroxide. However, all four nucleotides were approximately equally labelled as shown by autoradiography of nucleotides separated by polyethyleneimine thin-layer chromatography (Volckaert & Fiers, 1977) after complete hydrolysis with nuclease P1 of the labelled RNA eluted from the gel after electrophoresis (results not shown). The same result was obtained when both virions and RNA were purified quickly with a minimal number of steps, or when purification was more extensive and included electrophoresis of the RNA in 5 mM-methylmercuric hydroxide-containing agarose gels before 5′ end-labelling.

This apparent heterogeneity at the 5′ end could have resulted from degradation *in vitro* or *in vivo*, or could mean that functional virus RNA has such heterogeneity. The fact that the heterogeneity was not changed by modifying purification procedures argues against partial hydrolysis during purification. It was not possible to check for *in vivo* degradation by comparing virus purified from either young or old leaves because of insufficient space to grow the required number of plants in environmental chambers.

SBWMV RNA II differs from most plant virus RNAs in having no detectable 5′ cap or genome-linked protein. Except for satellite virus of tobacco necrosis (Leung *et al.*, 1979) most plant viruses have either a 5′ cap or a genome-linked protein (Atabekov & Morozov, 1979; Davies & Hull, 1982). The lack of a 5′ cap and lack of hybridization between SBWMV cDNA and tobacco mosaic virus RNA are further reasons for removing SBWMV from the tobamovirus group and establishing a new group, furovirus, as suggested by Shirako & Brakke (1984*a*). One tentative member of this new group, beet necrotic yellow vein virus, has RNAs with 5′ cap structures and 3′ poly(A) tracts (Putz *et al.*, 1983). Its inclusion in the proposed furovirus group is questionable.

These results support the hypothesis that the short rods that appear after several months in plants infected with SBWMV represent deletion mutants of RNA II. It was not possible to identify the site of the deletion because of heterogeneity at the 5′ end and resistance of the 3′ end to polyadenylation. The mutant RNA II seems to have an advantage over the WT RNA II in virus transmitted by leaf rubbing. The reason for survival of the WT RNA in the field is unknown.

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