Maize White Line Mosaic Virus Double-Stranded RNA, Replicative Structure, and In Vitro Translation Product Analysis

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ABSTRACT

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The possible involvement of subgenomic RNAs in maize white line mosaic virus (MWLMV) replication was investigated. In addition to full-length genomic double-stranded (ds) RNA, dsRNA with a "subgenomic" size (2.0 kb) was detected in extracts from MWLMV-infected corn plants. The viral RNA structures synthesized in vitro by a partially purified enzyme complex from MWLMV-infected corn leaves were similar to MWLMV replicative form (RF) and replicative intermediate form in electrophoretic behavior and ribonuclease sensitivity. Two major RFs (4.2 and 2.0 kb) and two minor RFs (1.2 and 0.8 kb) were found. The two major RFs corresponded to the two major dsRNAs found in MWLMV-infected corn plant extract. In vitro translation studies showed that MWLMV RNA directed the synthesis of four major polypeptides (26, 34, 35, and 50 kDa). The 34- and 35-kDa polypeptides reacted weakly with antiserum specific to the MWLMV virion. This result and those from western-blot analysis showed that the 35-kDa translation product was the MWLMV capsid protein. A model for MWLMV genome organization is proposed based on the comparison of features of MWLMV and the well characterized viruses carnation mottle, turnip crinkle, cucumber necrosis, and maize chlorotic mottle.

Maize white line mosaic virus (MWLMV) is an isometric (35 nm) plant virus with a single-stranded (ss), positive sense RNA genome of 1.25–1.65 $\times$ 10^6 (3.9–4.8 kb) and a capsid protein of 32–37 kDa (3,7,14). A satellite virus is associated with some isolates of MWLMV. The satellite virus of MWLMV (SV-MWLMV) (17 nm) contains an ssRNA of about 1.2 kb and a single coat protein with a molecular weight of about 24 kDa (8,23).

The genome of MWLMV has not been characterized with respect to its replication strategy, method of gene expression, or genome organization. To further characterize this virus, double-stranded (ds) RNA from MWLMV-infected corn plants was isolated and analyzed. The MWLMV RNA replicase activity in extracts of MWLMV-infected corn was tested and the in vitro replication products analyzed. In addition, the following proteins were analyzed: the in vitro translation products of MWLMV RNA; the immunoprecipitated in vitro translation products of MWLMV RNA; and proteins extracted from MWLMV-infected plants. A tentative model for MWLMV is proposed based on comparison with several well characterized RNA plant viruses.

MATERIALS AND METHODS

MWLMV purification and embryo-wounding corn plant inoculation with MWLMV. The purification procedure described by deZoeten et al (7) was used to isolate and purify MWLMV particles. Sweet corn (*Zea mays* L. 'Sunbeam') seeds were inoculated with MWLMV preparations (with or without SV-MWLMV), using an embryo-wounding technique (22). The inoculated seeds were planted in soil and maintained in a greenhouse under natural light conditions.

dsRNA isolation and analysis. MWLMV-infected corn leaves, with or without the associated SV, were frozen and ground to a powder in liquid nitrogen with a mortar and pestle. Nucleic acids were phenol-extracted and purified by chromatography on CF-11 cellulose columns (16), and were fractionated on a 1% agarose gel in electrophoresis buffer (40 mM Tris-HCl, pH 7.8, 20 mM sodium acetate, 1 mM EDTA). Double-stranded RNA isolated from tobacco mosaic virus- and cucumber mosaic virus-infected leaves were used as dsRNA molecular weight markers. Nucleic acids extracted from mock-inoculated corn leaves served as controls. Molecular hybridization analysis of dsRNA was done as described by Bar-Joseph et al (2) with [^{32}P]-labeled MWLMV cDNA as the probe. The cDNA probe was prepared as previously described (23).

Detection of viral RNA replicase activity in extracts of MWLMV-infected corn and analysis of the replicase assay products. Ten-day-old corn seedlings infected with MWLMV, with or without the SV, were used for this study. Mock-inoculated corn seedlings were used as controls. Corn leaves were homogenized by blending. After ultracentrifugation at 30,000 *g* for 30 min, the pellet-fraction (containing the viral replicase activity) was used to incorporate α-[^{32}P]-UTP into dsRNA (20). The radiolabeled RNA products from the replicase reactions were isolated by phenol extraction and concentrated by ethanol precipitation. These RNAs were analyzed by electrophoresis in 1.0% agarose gels in Tris-acetate-EDTA buffer and the [^{32}P]-labeled RNAs were detected by autoradiography as described below (12).

For the ribonuclease sensitivity tests, two samples from each replicase reaction were loaded onto the same agarose gel. After electrophoresis, the gel was cut in half, with each half containing identical samples. One half was soaked in 2$\times$ SSC (1$\times$ SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), and the other half was soaked in 0.1$\times$ SSC. Ribonuclease (RNase) A (2 μg/ml) was added to each half gel and both were incubated at 37 C for 30 min. After RNase digestion, both gel halves were dried onto Whatman filter paper at 60 C in vacuo and exposed to preflashed Kodak-X-AR film with an intensifying screen at −70 C.

In vitro translation and immuno-blot analysis. MWLMV RNA was extracted as previously described (17) and translated in vitro

in either a rabbit reticulocyte lysate or a wheat germ cell-free system (Promega, Madison, WI), with [^{35}S]-labeled methionine (>1,000 Ci/mmol, New England Nuclear, Boston, MA). Translation reactions were performed according to the manufacturer's instructions. The time course of the in vitro translation was performed by collecting samples for each time point from a single translation reaction, and these time course samples were mixed with sodium dodecyl sulfate (SDS)-buffer to stop the synthesis at each time point.

The translation products were analyzed by SDS-PAGE as described by Laemmli (11). After electrophoresis, the gel was fixed in 40% methanol, 10% glacial acetic acid, vacuum dried at 80 C for 1 h, and exposed to preflashed Kodak X-Omat AR film at −70 C.

The immunoprecipitation of the in vitro translation products described by Anderson and Blobel (1) was followed, using a 10% (w/v) suspension of formalin-fixed *Staphylococcus aureus* cells (PansorBin cells, CalBiochem. La Jolla, CA) as the immunoadsorbent (10). The antiserum to MWLMV, kindly provided by Drs. G. Boccardo and E. J. Lulkin, showed a titer of 1:512 as determined by Ouchterlony double diffusion test.

To determine if the antiserum reacts specifically for MWLMV, immuno-blot analysis was conducted following the method described by Towbin et al (19). The procedure for total plant protein preparation is very similar to that used for the in vitro replication experiment. Therefore, we used the 30,000 *g* pellet and supernatant fractions prepared for the replicase activity study (20) in the immuno-blot analysis. The same MWLMV antiserum was used for immunoprecipitation.

RESULTS

Isolation and detection of MWLMV dsRNA. The CF-11 cellulose chromatography fractions when analyzed by gel electrophoresis yielded a dsRNA preparation. Two major dsRNA species with estimated molecular weights of 4.2 and 2.0 kb, respectively (Fig. 1A, lane 2 and 1B, lane 1) were visible in the gel. In addition, up to 13 minor bands with estimated size ranges from 4.0 to 0.8 kb were observed when the sample was overloaded (Fig. 1B, lane 1). For the two major bands, the 4.2-kb band showed the expected molecular weight for the replicative form (RF) of genomic MWLMV RNA. The 2.0-kb dsRNA species could be the RF of a putative MWLMV subgenomic RNA. The minor bands (Fig. 1B, lane 1) could be either subgenomic RNAs or breakdown products. For the dsRNA extracted from MWLMV-infected leaves containing the SV, the amount of 2.0 kb of dsRNA was reduced (Fig. 1B, lane 2), and a dsRNA band with an estimated molecular weight of 1.2 kb was also present (Fig. 1B, lane 2). This 1.2-kb dsRNA corresponded to the RF of the SV-MWLMV RNA. No dsRNAs could be detected in healthy corn tissue extracts (Fig. 1B, lane 3). The above results were reproducible with different dsRNA preparations.

To determine whether the dsRNAs originated from MWLMV infection, the presence of MWLMV RNAs was analyzed by cDNA hybridization. The results showed that both of the major dsRNAs (4.2 and 2.0 kb) extracted from MWLMV-infected leaves (without the SV) hybridized with MWLMV cDNA probe (Fig. 1C, lane 2).

Viral RNA replicase activity in MWLMV-infected plant extract and replicase product analysis. The 10,000–30,000 *g* pelleted subcellular fraction from RNA plant virus-infected plants has been shown to contain viral replicase activity (20). The 30,000 *g* pellet fraction from mock-inoculated corn leaves synthesized only low molecular weight, heterodisperse RNAs (Fig. 2A, lane 1). The major RNA products synthesized from the 30,000 *g* pellet fraction from MWLMV-infected leaves (Fig. 2A, lane 2) were: high molecular weight, heterodisperse RNAs that had the properties of replicative intermediate (RI) (see below); an RNA species (about 4.2 kb) with an electrophoretic mobility equal to that of full-length MWLMV dsRNA, which had the properties of the RF of MWLMV genomic RNA (see below); an RNA species (about 2.0 kb) with electrophoretic mobility equal to that of subgenomic MWLMV dsRNA, which might be the RF of the subgenomic RNA; two less intense, lower molecular weight RNAs that were about 1.2 and 0.8 kb, respectively; and very low molecular weight, heterodisperse RNAs, which migrated at or near the front, and are probably products of the plant RNA dependent RNA polymerase (20).

The ssRNA or dsRNA nature of these viral-specific products was determined by the sensitivity of these RNAs to RNase A, which can digest both ssRNA and dsRNA at low salt concentrations, but is specific for ssRNA at high salt concentrations (e.g., 2× SSC) (20). As has been shown for the RF of tobacco mosaic virus (20), treatment with RNase A caused the disappearance of the RI-like RNA, but had little effect on RF-like RNAs

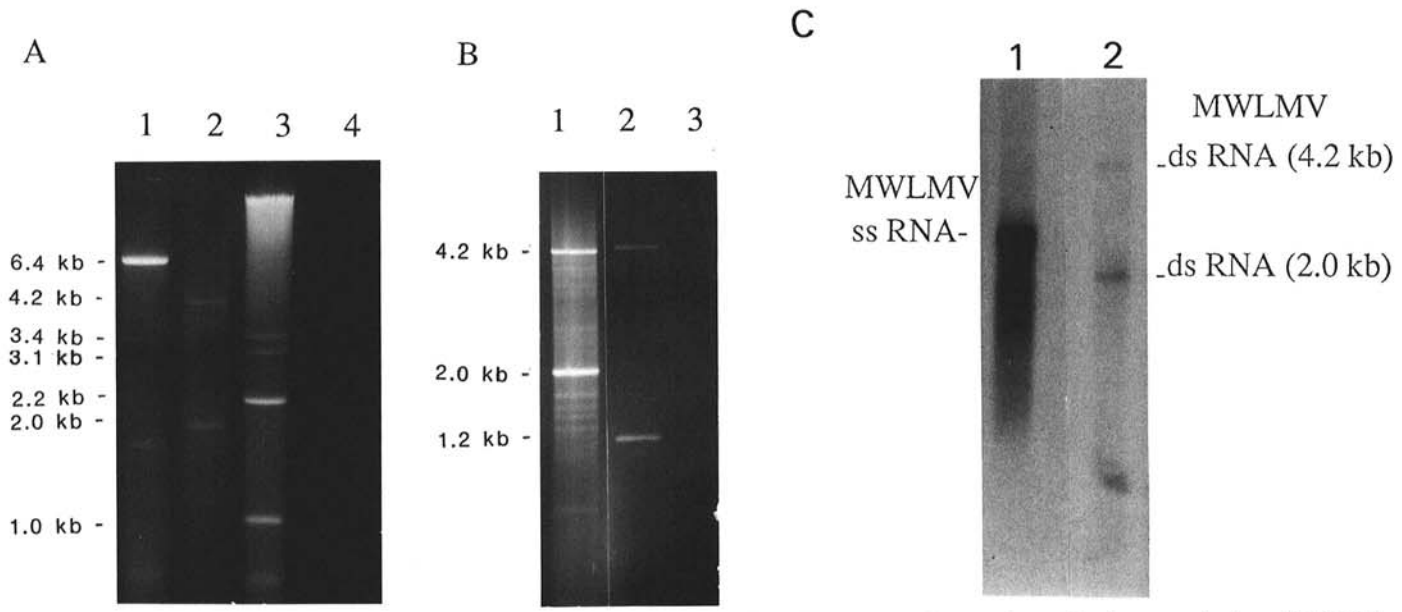

Fig. 1. Non-denaturing 1.0% agarose gel electrophoresis of double-stranded (ds) RNA isolated from maize white line mosaic virus (MWLMV)-infected corn (with and without the satellite virus (SV) present). **A,** lane 1, dsRNA from tobacco mosaic virus-infected leaves; lane 2, dsRNA from MWLMV-infected leaves; lane 3, dsRNA from cucumber mosaic virus-infected leaves; lane 4, extract from mock-inoculated leaves. **B,** lane 1, dsRNA from MWLMV-infected leaves; lane 2, dsRNA from MWLMV- and SV-MWLMV-infected leaves; lane 3, extract from mock-inoculated leaves. **C,** hybridization of dsRNA blots with labeled MWLMV cDNA probe. Lane 1, single-stranded MWLMV RNA; lane 2, dsRNA from MWLMV-infected plants.

(Fig. 2B, lane 2). At low salt concentrations (0.1× SSC), virtually all RNAs were digested by RNase A (Fig. 2B, lanes 1 and 2).

In vitro translation and western-blot analysis. MWLMV RNA, either extracted from virion or from infected plants, were used separately to direct the synthesis of viral-specific polypeptides in a cell-free in vitro translation system. MWLMV RNA efficiently directed the synthesis of four polypeptides in both the wheat germ and rabbit reticulocyte cell-free systems (Fig. 3A, lane 2, and 3B, lane 2, respectively). The translation products detected after SDS-PAGE were polypeptides of 50, 35, 34, and 26 kDa in molecular weight. Minor amounts of polypeptides greater than 80 kDa were only seen using the reticulocyte lysate (Fig. 3B, lane 2). The 35-kDa polypeptide was identical in size to the MWLMV capsid protein subunit (Fig. 3A, lane 2, and 3B, lane 2). The 26-kDa polypeptide showed a stronger signal in the wheat germ system than in the rabbit reticulocyte system (Fig. 3A, lane 2 vs. 3B, lane 2). Besides the above four polypeptides, some small protein products (between 15 and 24 kDa) were observed (Fig. 3C, lanes 4 and 5). To determine whether post-translational processing is involved in MWLMV gene expression, a time course analysis of the in vitro translation products was conducted. The results showed that the amount of each translation product kept increasing until about 1 h (Fig. 3C), and the ratios between them remained the same up to 3 h (data not shown). No higher molecular weight proteins were synthesized at any sampling time.

Immunoprecipitation experiments were used to determine which polypeptide was the capsid protein. The results showed that the 35-, 34-, 24-, 22-, and 20-kDa polypeptides reacted with an antiserum specific for MWLMV (Fig. 4A, lane 2). The 35-kDa polypeptide generated by in vitro translation co-migrated with the MWLMV capsid protein subunit. In immunoprecipitation tests this 35-kDa protein reacted with an antiserum specific for MWLMV virions. The 34-, 24-, 22-, and 20-kDa polypeptides may be partially synthesized or degraded products of a 35-kDa capsid protein subunit, because only the 35-kDa subunit was found in MWLMV virions. Immuno-blot analysis of disrupted virions and proteins from fractionated infected tissues also showed that only the 35-kDa protein reacted with an antiserum specific for MWLMV (Fig. 4B, lanes 1–3). This antiserum did not react with plant proteins (Fig. 4B, lane 4).

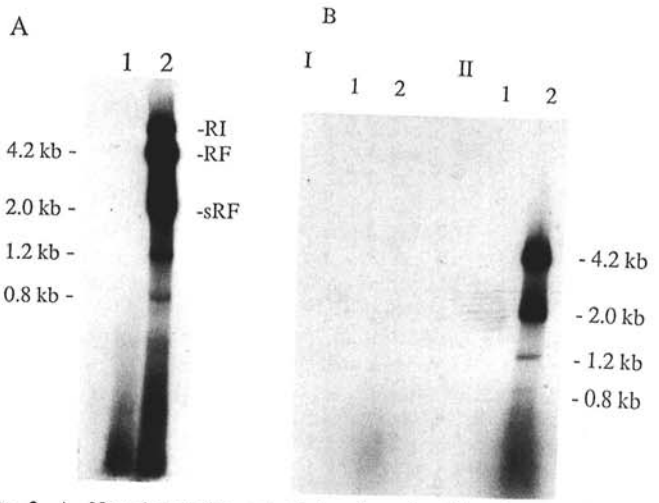

Fig. 2. A, Non-denaturing gel electrophoresis of in vitro synthesized replicase products. Radiolabeled RNAs were synthesized in vitro and separated on 1.0% agarose gels as described in Materials and Methods. Lane 1, RNA products from mock-inoculated leaves; lane 2, replicase products from maize white line mosaic virus (MWLMV)-infected leaves (no satellite virus-MWLMV). **B,** ribonuclease treatment of in vitro synthesized replicase products. In vitro labeled RNAs were incubated with ribonuclease (RNase) A and analyzed by 1.0% agarose gel electrophoresis, and autoradiographed as described in Materials and Methods. I, after electrophoresis the gel was treated with RNase A in 0.1× SSC (1× SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0). Lane 1, RNA products from mock-inoculated leaves; lane 2, replicase products from MWLMV-infected leaves. II, after electrophoresis the gel was treated with RNase A in 2× SSC. Lane 1, RNA products from mock-inoculated leaves; lane 2, replicase products from MWLMV-infected leaves.

DISCUSSION

By analyzing either ssRNA or dsRNA, we should be able to determine whether plant viruses produce subgenomic RNAs. However, some plant viruses do not encapsidate subgenomic RNAs into virions. In many cases, subgenomic RNAs either present in total RNA extracts of diseased tissue or extracted from polyribosomes are masked by ribosomal RNA shadowing (17). This was the case with MWLMV (data not presented).

A characteristic of RNA plant virus replication is the presence of dsRNAs in diseased tissue. These include dsRNAs of the genomic as well as of the subgenomic RNAs. Thus, dsRNA analysis has become a standard method for determining the number and size of subgenomic RNAs of plant viruses and has been used extensively (2).

Besides the MWLMV genomic size RNA (4.2 kb), subgenomic size RNAs (2.0, 1.2, and 0.8 kb) were found both in MWLMV dsRNA preparations and in in vitro replication studies, although no correlation was made between these RNAs and the protein products translated in vitro. MWLMV RNA is a very poor messenger RNA (mRNA) for in vitro translation.

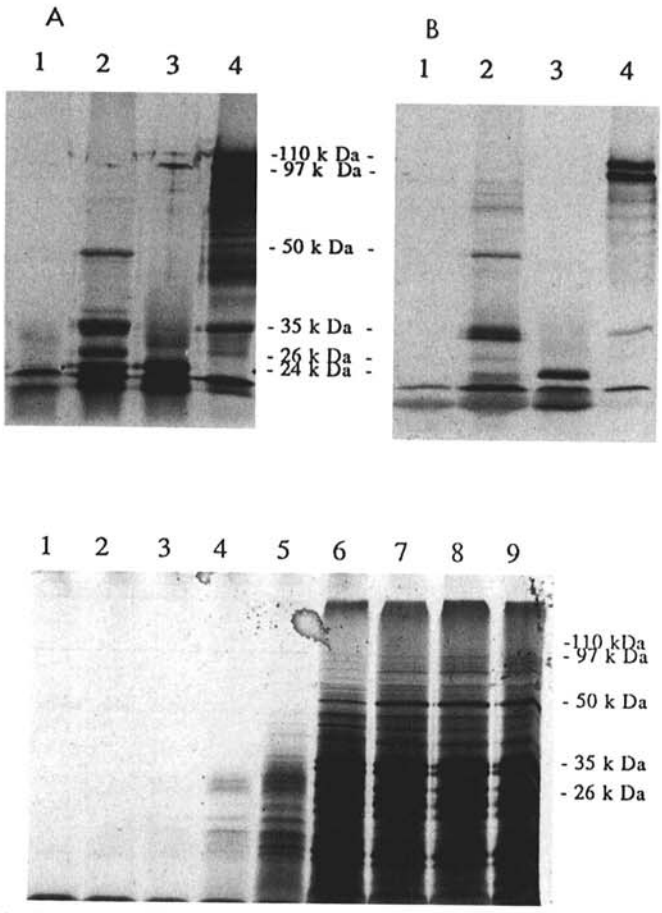

Fig. 3. A, autoradiogram of a 10% polyacrylamide-0.1% sodium dodecyl sulfate (SDS) gel used to analyze cell-free translation products from viral RNAs in the wheat germ translation system: 1) no RNA; 2) maize white line mosaic virus (MWLMV) RNA; 3) satellite virus (SV)-MWLMV RNA; 4) brome mosaic virus RNA. **B,** autoradiogram of a 10% polyacrylamide-0.1% SDS gel used to analyze cell-free translation products from viral RNAs in the rabbit reticulocyte lysate translation system: 1) no RNA; 2) MWLMV RNA; 3) SV-MWLMV RNA; 4) brome mosaic virus RNA. **C,** time course analysis of MWLMV RNA in vitro translation products in a rabbit reticulocyte lysate system. Samples were electrophoresed through 12.5% polyacrylamide-0.1% SDS gels and fixed for autoradiography. The time points were 15 s (1), 30 s (2), 1 min (3), 2 min (4), 4 min (5), 8 min (6), 16 min (7), 32 min (8), and 64 min (9).

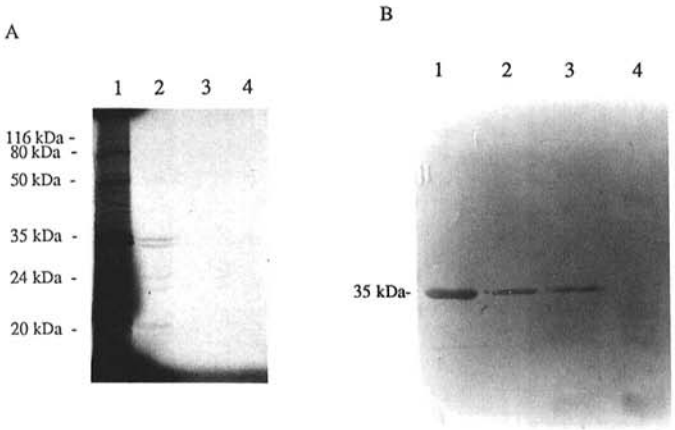

Fig. 4. A, autoradiogram of a 12.5% polyacrylamide-0.1% sodium dodecyl sulfate (SDS) gel used to analyze reticulocyte lysate cell-free translation products from the viral RNAs, before (1) and after (2–4) immunoprecipitation. 1) maize white line mosaic virus (MWLMV) RNA; 2) MWLMV RNA with MWLMV antiserum; 3) MWLMV RNA with preimmunization serum; 4) MWLMV RNA with water. **B,** western blot of electrophoresed proteins probed with a MWLMV-specific antiserum. 1) Capsid protein from purified MWLMV virion; 2) proteins from 30,000 *g* pellet from an extract of a MWLMV-infected corn plant (the one used as the crude MWLMV replicase preparation); 3) proteins from the 30,000 *g* supernatant, made from the same source as in 2; 4) proteins from the 30,000 *g* pellet from an extract of a mock-inoculated corn plant.

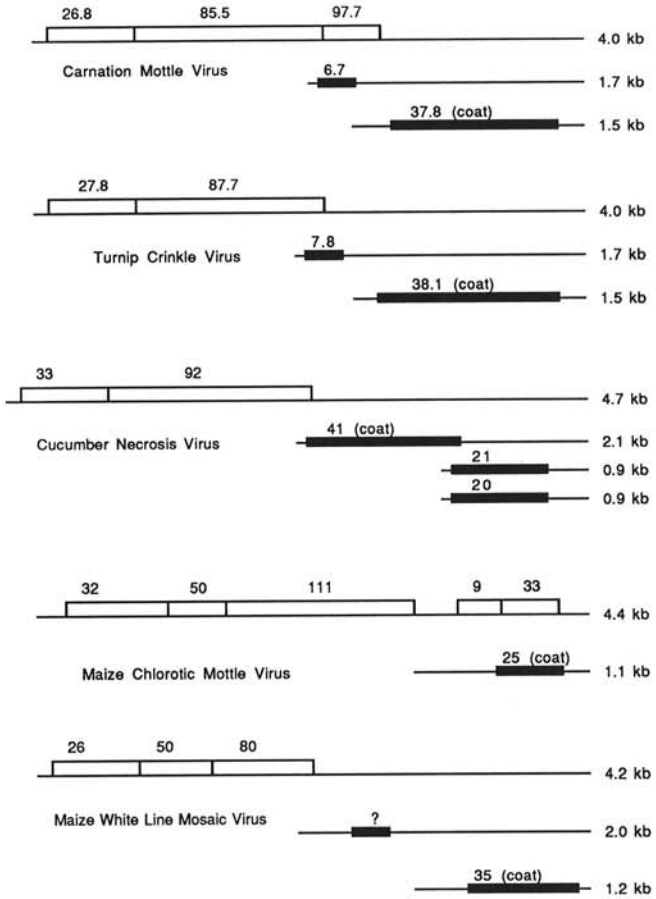

Fig. 5. Models comparing the organization and expression of carnation mottle virus (CarMV), turnip crinkle virus (TCV), cucumber necrosis virus (CNV), maize chlorotic mottle virus (MCMV), and maize white line mosaic virus (MWLMV) genomes. The relative size and positions of mapped subgenomic RNAs are depicted as lines. The possible open reading frames potentially expressed from the RNAs are indicated by the white or black boxes. The approximate sizes of the encoded gene are indicated above the boxes, and sizes of the genomic and subgenomic RNAs are given at the end of each line. The maps of CarMV, TCV, MCMV, and CNV were adapted from the models presented previously (4,13,18). The MWLMV map was drawn based on data obtained in this study.

This may be due to the very small amount of subgenomic RNAs that is present in the MWLMV RNA population. The result of time course analysis of in vitro translation showed that the ratio of translation products remained the same for up to 3 h. No significant amount of higher molecular weight proteins were synthesized at any sampling time. There is no evidence to support the involvement of polypeptide processing in MWLMV gene expression. In vitro translation of sucrose gradient-fractionated MWLMV RNA also suggests that the 35-kDa coat protein of MWLMV was produced from subgenomic RNA and not by polypeptide processing (21).

The properties of MWLMV are very similar to those of so-called "small RNA plant viruses," such as carnation mottle virus (CarMV) (5,6), turnip crinkle virus (TCV) (4), tobacco necrosis virus (TNV) (15), maize chlorotic mottle virus (MCMV) (13), cucumber necrosis virus (CNV) (18), and tomato bushy stunt virus (9). They all have small isometric particles (30–35 nm in diameter) with a single component, positive sense RNA genome of 4.0–4.8 kb. They all express the coat protein gene from subgenomic RNAs. In addition to the similarities in physical features between MWLMV and the well characterized small plant RNA viruses, MWLMV shows similarities in its in vitro translation products and subgenomic RNA patterns. For example, MWLMV RNA produced a 26-kDa polypeptide in the cell-free translation system (Fig. 3A, lane 2), which may correspond to the small (26.8–33 kDa) polypeptide that the other small viruses (CarMV, TCV, CNV, and MCMV) encode near the 5′ terminus of their genomes (Fig. 5). The 80-kDa polypeptide that MWLMV produced in the cell-free translation system may correspond to the read-through product of the 26.8- to 33-kDa polypeptide from those small RNA viruses mentioned above (Fig. 3B, lane 2 and Fig. 5). The 50-kDa protein product of MWLMV (Fig. 3A, lane 2) may be a read-through product, as is the case for a 50-kDa polypeptide in MCMV (13), or it may be an internal initiation product, as has been observed for a 50-kDa polypeptide synthesized during TNV RNA-directed cell-free translation (15).

When comparing the subgenomic RNA patterns of small RNA plant viruses with MWLMV, CarMV and TCV produce major subgenomic RNAs of 1.7 and 1.5 kb (4–6), and CNV produces a 2.1-kb and two 0.9-kb subgenomic RNAs (18) (Fig. 5), whereas MWLMV produces a major putative subgenomic RNA (2.0 kb) (Fig. 1A, lane 2) and some smaller possible subgenomic RNAs (1.2 and 0.8 kb dsRNAs, Fig. 2A, lane 2). CarMV, TCV, and TNV all use the smallest subgenomic RNA to express the coat protein gene (4–6, 15), whereas CNV uses the 2.1-kb subgenomic RNA to express the coat protein gene (18). For MWLMV, the coat protein gene could potentially be expressed from either the 2.0-kb or the 1.2-kb subgenomic RNA.

Although there is not sufficient evidence to present a definite genome organization for MWLMV, a tentative model for the genome organization of MWLMV is proposed based on the similarities between CarMV, TCV, CNV, MCMV, and MWLMV (Fig. 5). In this model, the MWLMV genomic RNA functions as an mRNA for translation of a 26-kDa protein and read-through products of 50 kDa and 80 kDa. The 1.2-kb subgenomic RNA is proposed to function as an mRNA to produce the capsid protein (35 kDa). At this time it is not certain what protein is encoded by the 2.0-kb subgenomic RNA, although some low molecular weight polypeptides (smaller than 20 kDa) were produced in the cell-free translation system (Fig. 3).

Although the coat protein of MWLMV is presumably expressed from a subgenomic RNA in vivo, the 35-kDa coat protein is one of the main in vitro translation products of MWLMV genomic RNA (Fig. 3A, lane 2). This suggests that MWLMV RNA may act as a polycistronic messenger in vitro, as is the case for the in vitro translation of TNV RNA (15).

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