Genome Properties of Bamboo Mosaic Virus

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ABSTRACT

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Bamboo mosaic virus (BaMV), isolated from infected green bamboo (*Bambusa oldhamii*) in Taiwan, is a flexuous, rod-shaped virus. The genome of BaMV is a single-stranded plus-sense RNA, M_r 2.1 × 10^6, encapsidated with capsid protein subunits of M_r 28K. Sodium dodecyl sulfate-immunodiffusion and western blot analysis showed that BaMV is serologically unrelated to the tested potexviruses and other viruses. BaMV RNA, fractionated or unfractionated, directed the synthesis of one prominent high molecular weight product, M_r 160K, together with several polypeptides of smaller sizes when translated in rabbit reticulocyte lysate. Immunoprecipitation of the in vitro translation products failed to reveal the synthesis of viral capsid protein. No encapsidated subgenomic RNA could be detected by northern hybridization with cDNA transcribed from genomic RNA. However, northern blots of RNAs extracted from infected barley protoplasts detected two major subgenomic RNAs of 2.0 and 1.0 kb in size. All of these properties suggest that BaMV is indeed a member of the potexvirus group.

Bamboo mosaic commonly occurs in the plantation of bamboo species with rhizomes of the pachymorph type in Taiwan (24,25). It has remained a serious problem for the bamboo industry on the island. Bamboo mosaic virus (BaMV, proposed by Hull et al (17), which was previously acronymized as BoMV), a tentative member of the potexvirus group, is regarded as the causal agent (23,25). The tentative grouping of this virus with the potexvirus group is primarily based on virus morphology and some other properties (19). Further evidence was the cytological characteristics that virions formed aggregates in the cytoplasm of BaMV-infected bamboo cells (18,24). However, BaMV is unique among the potexviruses in the induction of electron-dense crystalline bodies (EDCBs), which are serologically unrelated to BaMV capsid protein. Moreover, in infected cells, EDCBs are synthesized earlier than the capsid protein, and thus, EDCBs can serve as infection initiation markers (24).

In this report, we determined the molecular weight of the capsid protein and nucleic acid, the serological relationship with other potexviruses, the translation strategy of the BaMV genome, and the presence of subgenomic RNAs in BaMV-infected cells. All the results suggest that BaMV is indeed a member of the potexvirus group. A preliminary report on in vitro translation has been given (16).

MATERIALS AND METHODS

Viruses. BaMV was isolated from infected green bamboo (*Bambusa oldhamii* Munro). Following three successive single lesion transfers on *Chenopodium quinoa*, the virus was propagated in barley (*Hordeum vulgare* L. 'Larker') (24). Potato virus X (PVX) and tobacco mosaic virus (TMV) were received from Y. C. Lu (Department of Plant Pathology, National Chung Hsing University, Taiwan 400). Both the ND18 strain of barley stripe mosaic virus (BSMV) and brome mosaic virus (BMV) were gifts from M. K. Brakke (Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583). The M48 strain of cucumber mosaic virus (CMV) (15) was propagated in tobacco.

Virus purification and preparation of antiserum. Purification of BaMV and preparation for antiserum against gel-eluted BaMV capsid protein (Anti-BaMV-CP serum) were described previously (24). Anti-BaMV-CP serum had a titer of 1/16 as determined by immunodiffusion tests.

Sodium dodecyl sulfate (SDS) immunodiffusion test. Procedures for immunodiffusion tests were described by Purcifull and Batchelor (32). Antisera against cassava common mosaic virus (CsCMV) (36) and nandina mosaic virus (NaMV) (37) were kindly provided by F. W. Zettler (Institute of Food and Agricultural Sciences, University of Florida, Gainesville, 32611). Antisera against cymbidium mosaic virus (CyMV) and PVX were from C. A. Chang (Taiwan Agricultural Research Institute, Taiwan 413) and Y. C. Lu, respectively.

Polyacrylamide gel electrophoresis of virion protein. Purified BaMV virions were disrupted in protein sample buffer (60 mM Tris-HCl, pH 8.8, 2% SDS, 2 mM EDTA, 5 mM DTE, and 10% Ficoll) (22) and analyzed in a 12% polyacrylamide gel (PAG) using a discontinuous buffer system (21). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Molecular weight markers used were purchased from Pharmacia LKB (Biotechnology Inc., Piscataway, NJ 08855): phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase 30,000; soybean trypsin inhibitor 20,100; α-lactalbumin, 14,400.

Western blotting. Western blotting analysis was conducted as described previously (26). Protein samples, 0.1 μg each, resolved in a 12% PAG after electrophoresis, were electrotransferred to a nitrocellulose sheet. Diluted anti-BaMV-CP serum (1:1000) was used as a primary antibody followed by staining with gold-labelled goat antirabbit IgG complexes (13).

Virion RNA isolation and gel electrophoresis. RNAs were isolated from purified virions disrupted in a solution containing 2% SDS, 10 mM NaH_2PO_4, 20 mM Na_2HPO_4, and 1 mM EDTA at 60 C for 5 min, followed by conventional phenol-chloroform extraction and ethanol precipitation. Gel electrophoresis to estimate the RNA molecular weight was performed as described (14). RNA was denatured by 6% formaldehyde, electrophoresed on a 1.5% agarose gel, and visualized by staining with 0.5 μg/ml ethidium bromide. To obtain full-length genomic RNA, the viral RNA samples were fractionated by linear sucrose density gradient centrifugation (14).

In vitro translation and immunoprecipitation. Translation of BaMV and PVX RNAs in rabbit reticulocyte lysate (RRL, purchased from Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD 20877) and immunoprecipitation

of translation products were performed as described (14). The translation products and immunoprecipitates were analyzed on a 8–20% linear-gradient SDS-PAG and detected by fluorography (8).

Protoplast isolation and infection with BaMV RNA. Barley protoplasts were isolated from 7-day-old seedlings according to the method of Loesch-Fries & Hall (27) modified by Kroner et al (20). Approximately 5×10^5 protoplasts were inoculated with 2 μg of BaMV RNA by the polyethylene glycol procedure (20) and incubated in a growth chamber at 24 C under 24-h daily exposure to 50 $\mu Em^{-2}sec^{-1}$ illumination. Twenty-four hours after infection, protoplasts were harvested for northern blot analysis.

Northern blot analysis. For northern blot analysis, total nucleic acids were extracted from virions, infected bamboo leaves, or transfected protoplasts. They were denatured in the presence of glyoxal, separated in a 1% agarose gel, and blotted to the H-Bond hybridization membrane (Amersham International, Amersham, Buckinghamshire, England HP7 9NA) by a procedure described by Kroner et al (20). For the detection of RNA synthesis, a cDNA probe was synthesized by priming with random primer (New England Biolabs, Inc., Beverly, MA 01915) using BaMV RNA as a template in the presence of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) (29). The reaction mixture contained 2 μg RNA and typically gave a yield of 1–2 $\times$ 10^8 cpm radioactive cDNA. Prehybridization and hybridization conditions for northern blot analysis followed the description by Kroner et al (20).

RESULTS

Serological relationship of BaMV with other potexviruses. In immunodiffusion test, purified BaMV virion antigens only reacted with homologous antiserum but failed to react with antisera against CsCMV, NaMV, CyMV, PVX, and normal rabbit serum as shown in Fig. 1.

Gel electrophoresis of viral protein and western blotting. Electrophoresis of SDS-disrupted purified BaMV in polyacrylamide gel revealed only one protein species with an average M_r of 28K. Fig. 2A shows that BaMV capsid protein migrated faster than that of PVX but slower than capsid protein of BSMV, TMV, and BMV in a 12% polyacrylamide gel.

In western blotting analysis, a 1:1,000 dilution of anti-BaMV-CP serum reacted specifically only with the homologous antigen but not with the capsid protein of other viruses tested (Fig. 2B).

Gel electrophoresis of viral nucleic acid. BaMV RNA extracted from virions and denatured with formaldehyde was electrophoresed in a 1.5% agarose gel. A single species of RNA was observed, with a M_r estimated at 2.1 $\times$ 10^6, corresponding to 6,400 nucleotides, using TMV, BMV, and PVX RNAs as standards (Fig. 3). No other discrete RNA species or heterogeneous low-molecular weight components were detected in either the unfractionated or fractionated samples of BaMV RNA (lanes 3 and 4).

In vitro translation and immunoprecipitation. In order to test the template activity, BaMV RNA was translated in RRL. The full-length BaMV RNA directed the synthesis of several high molecular weight products as revealed on SDS-polyacrylamide gels. Among them, the most prominent one displayed a M_r of about 160K; there were also several products whose molecular weights were intermediate between l60K and the 28K capsid protein (Fig. 4A, lanes 2 and 3). There was little or no difference in the translation products from full-length or total unfractionated BaMV RNA (Fig. 4A, lanes 2 and 3). The translation products of BaMV were very similar to those obtained with PVX RNA (Fig. 4B, lane 2). Endogenous mRNA present in RRL synthesized one polypeptide having a M_r of 42K as shown in Fig. 4A, lane 1.

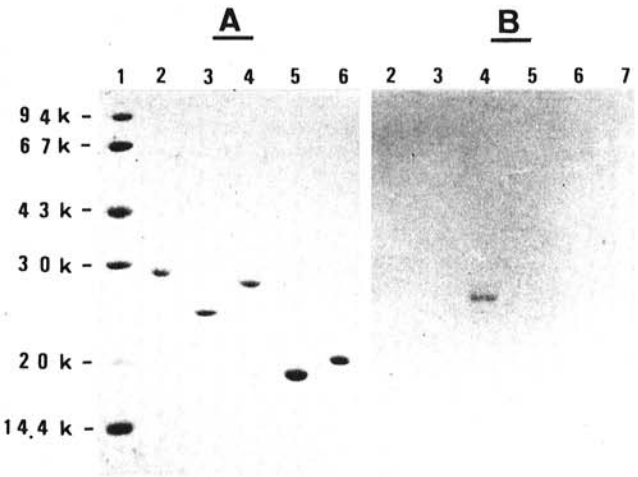

Fig. 2. A, SDS-polyacrylamide gel (12%) electrophoresis of virion proteins from purified preparations of PVX (lane 2), BSMV (lane 3), BaMV (lane 4), TMV (lane 5), and BMV (lane 6). Lane 1 is protein markers, from top to bottom: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin. The gel was stained with Coomassie Brilliant Blue R-250. **B,** Immunogold staining of western blot of same protein samples as in **A** (except without lane 1). Proteins in the gel were electro-transferred to nitrocellulose paper using Polyblot (American Biometics, Emeryville, CA 94608). The protein blot was reacted first with rabbit anti-BaMV-CP serum, followed by gold-labelled goat anti-rabbit IgG.

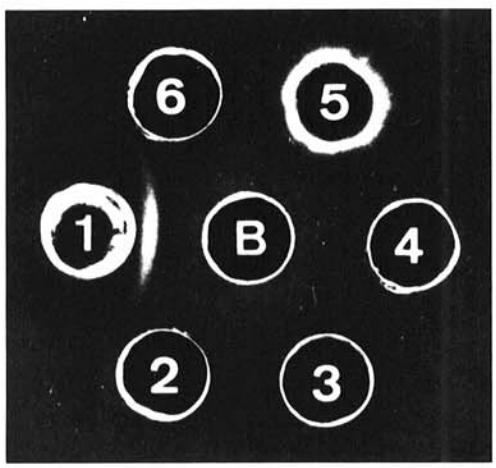

Fig. 1. Reactions in sodium dodecyl sulfate-immunodiffusion test of purified BaMV virions (**B,** 2.5 μg in the center well) to antisera against 1) BaMV, 2) CsCMV, 3) NaMV, 4) CYMV, 5) PVX, and 6) normal rabbit serum.

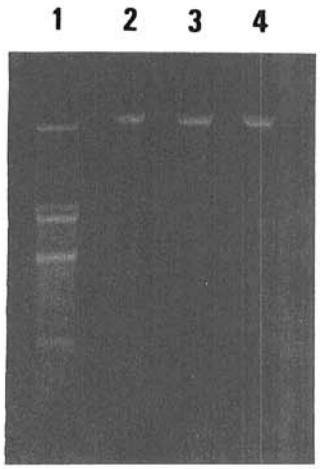

Fig. 3. RNA patterns of virion preparations in a 1.5% agarose gel. Lane 1, TMV and BMV RNAs; lane 2, PVX RNA; lane 3, unfractionated BaMV RNA; lane 4, fractionated BaMV RNA. The RNA samples were denatured by 6% formaldehyde and electrophoresed in agarose gel. One μg of RNA was loaded for each sample.

To identify the translation products of BaMV RNA, an immunoprecipitation assay for capsid protein was performed. No proteins translated from BaMV RNA were precipitated with anti-BaMV-CP serum (Fig. 4A, lanes 4 and 5) or with preimmune serum (not shown). These results indicate that none of the polypeptides synthesized in vitro are BaMV capsid protein or antigenically related to it. They are possibly nonstructural proteins.

Detection of subgenomic RNA. In the preliminary studies, total RNAs were extracted from healthy and BaMV-infected bamboo leaves. By using cDNA of the full-length BaMV RNA as a probe, BaMV genomic RNA was detected in infected tissues but not in uninfected tissues (not shown). Neither was any subgenomic RNA detected. In the northern blots of virion RNA, there was only one discrete hybridizing band detected (Fig. 5, lane 3). The results remained unchanged even when the film was purposely overexposed ten times longer than usual (data not shown).

When total nucleic acids were extracted from inoculated and mock-inoculated barley protoplasts 24 h after inoculation and probed with BaMV cDNA, three BaMV-specific RNA species were observed (Fig. 5, lane 1). The largest RNA (6.4 kb) comigrated with BaMV genomic RNA. Two smaller RNAs, about 2.0 kb and 1.0 kb in size, were also easily detected. In addition, there were some other minor RNA species, 4.6 kb, 3.0 kb, and 1.7 kb in size, present in the infected protoplasts (lane 1). No RNA species in extracts prepared from mock-inoculated protoplasts hybridized with the cDNA probe (lane 2).

DISCUSSION

In this study, we have demonstrated that the genome of BaMV contains a single-stranded positive-sense RNA based on template activity and northern hybridization studies. Two lines of evidence show that the genome of BaMV consists of a single RNA molecule. First, BaMV RNA, fractionated or unfractionated, migrated as a single species on electrophoresis after denaturation with formaldehyde (Fig. 3, lanes 3 and 4). Second, only one RNA species extracted from virions hybridized with ^{32}P-labelled cDNA that was copied from full-length genomic RNA (Fig. 5, lane 3). This RNA had a size corresponding to 6,400 nucleotides and was encapsidated with protein subunits of M_r 28K. Molecular weights of the nucleic acid and protein subunit of BaMV as determined above are in good agreement with the reported values for potexviruses thus far, namely, 6–7 kb for the genomic RNA and 23–28 kDa for the capsid protein subunits (19,30). However, there was only a distant serological relationship and little RNA homology was noted among members of the potexviruses (7,10,19). In this regard, BaMV is thus not unique in having no serological relationship with other potexviruses, such as CsCMV, CyMV, NaMV, PVX (Fig. 1), papaya mosaic virus (PMV), and clover yellow mosaic virus (CYMV) (23).

In a RRL cell-free translation system, BaMV RNA directed the synthesis of one major polypeptide with M_r 160K, as well as a number of minor ones with smaller molecular weights, but not the capsid protein (Fig. 4). These results are consistent with a model in which the genomic RNA of most potexviruses encodes a large nonstructural protein (M_r 145–182K) but fails in in vitro systems to direct the synthesis of any significant amount of capsid protein as observed with PVX (2,31,33,35), daphne virus X (DVX) (12), foxtail mosaic virus, and viola mottle virus (6). However, a number of potexviruses display divergence in the expression of their capsid proteins in vitro. Genomic RNAs of CYMV (6), narcissus mosaic virus (NMV) (28), and PMV (4) are able to direct the synthesis of their capsid protein in vitro and this ability has been shown to be the consequence of nuclease activity. Although BaMV RNA failed to generate active subgenomic RNA in a RRL system, the genome organization is probably analogous to that of most of the potexviruses, i.e. the gene for the large nonstructural protein (M_r 160K) being located at the 5′ terminus and that for capsid protein lying toward the 3′ end. The expression of capsid protein gene of BaMV may require the formation of subgenomic capsid protein mRNA, rather than by initiation at the internal site.

In regard to the presence of subgenomic RNAs of BaMV, we have demonstrated that barley mesophyll protoplasts infected with BaMV RNA contained two major BaMV-specific RNA species, 2.0 and 1.0 kb, in addition to the 6.4 kb genomic RNA species (Fig. 5, lane 1). However, the detection was not successful when

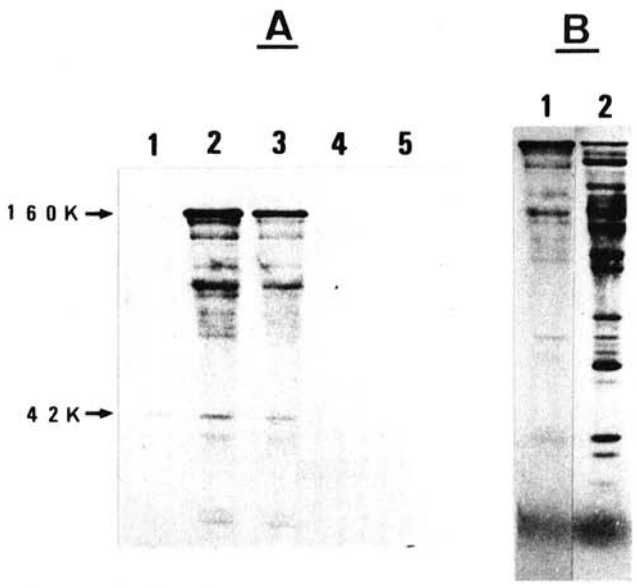

Fig. 4. Analysis of the in vitro translation products encoded by BaMV and PVX RNAs. RNAs were translated in rabbit reticulocyte lysate (RRL) supplemented with ^{35}S-methionine. Translation products were resolved in a SDS 8–20% polyacrylamide gel and detected by fluorography. **A,** Total translational products directed by endogenous mRNA (lane 1), unfractionated BaMV RNA (lane 2), and full-length BaMV RNA (lane 3). Lanes 4 and 5 are as in lane 2 and 3, respectively, after immunoprecipitation with anti-BaMV-CP serum. **B,** Comparison of the in vitro translation products of BaMV RNA (lane 1) and PVX RNA (lane 2).

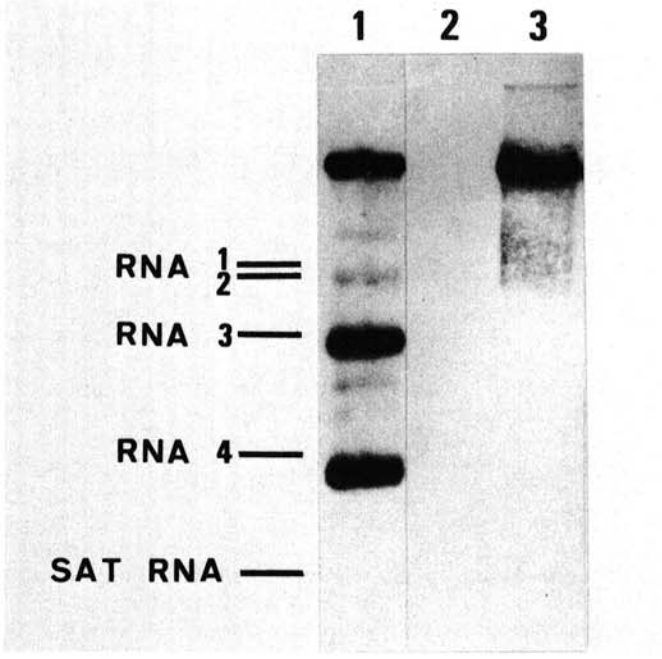

Fig. 5. Northern blot analysis of RNAs isolated from BaMV-inoculated (lane 1) and mock-inoculated (lane 2) barley protoplasts at 24 h postinoculation. Twenty nanograms of BaMV RNA was loaded in lane 3. The blot was probed with ^{32}P-labelled cDNA transcribed from BaMV RNA template. RNAs from M48 strain of CMV (15) were used as size standards.

total RNA was extracted from infected bamboo leaves. Potato protoplasts infected with PVX accumulated several subgenomic RNA species (1). Dolja et al (9) also reported that PVX-infected leaf cells displayed two major polyadenylated subgenomic RNAs of 0.9 kb and 2.1 kb in length. Furthermore, the 0.9 kb RNA has been shown to be the mRNA for the capsid protein. Subgenomic RNAs of other potexviruses, ranging from 0.8 to 1.0 kb, that encode capsid protein were also reported for CYMV (5,6), DVX (12), NMV (28,34), and white clover mosaic virus (WClMV) (11). However, for NMV and the M isolate of WClMV, subgenomic RNAs are efficiently encapsidated. We have obtained no evidence for any encapsidated subgenomic RNAs in BaMV viral preparations (Fig. 5, lane 3). Although the function of two major subgenomic RNAs of BaMV is unknown, we may expect that the 1.0 kb RNA, like that associated with other potexviruses, is responsible for the expression of the capsid protein. From our preliminary sequence data, BaMV contains five open reading frames, as most potexviruses do (Hsu et al, *unpublished*). Subgenomic RNA of 2.0 kb in length may correspond to transport protein gene as reported for WClMV (3).

All the properties of BaMV described above are consistent with those of typical potexviruses; therefore, BaMV should be classified as a member of this virus group.

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