Purification, Properties, and Diagnosis of Banana Bract Mosaic Potyvirus and Its Distinction from Abaca Mosaic Potyvirus

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ABSTRACT

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Using biochemical, serological, and cytopathological evidence, we have confirmed that banana bract mosaic virus (BBrMV) is a distinct member of the family *Potyviridae*. Virions of a Philippine isolate of BBrMV were purified from field-infected banana cv. Cardaba. Particles were approximately 725-nm long, banded at a density equivalent to 1.29 to 1.31 g/ml in cesium chloride equilibrium gradients, and had an $A_{260/280}$ of 1.17. Yields of about 4 mg/kg were obtained from fresh or frozen leaf midrib or lamina tissue. Three major protein species with sizes of 31, 37, and 39 kDa were resolved from dissociated virions, and all reacted specifically with polyclonal antibodies to BBrMV. Infected leaf cells contained typical pinwheel inclusions. Virus-specific cDNA was amplified from field samples by reverse transcription–polymerase chain reaction (RT-PCR) assay using potyvirus degenerate primers. In plate-trapped antigen–enzyme-linked immunosorbent assay (ELISA), weak serological relationships were demonstrated between BBrMV and other members of the family *Potyviridae*, including abaca mosaic (AbaMV), dasheen mosaic, maize dwarf mosaic, sorghum mosaic, sugarcane mosaic, and wheat streak mosaic viruses. Despite similarities in the symptoms caused by the two viruses, AbaMV was serologically distinct from BBrMV and reacted only weakly, or not at all, with BBrMV antibodies in double-antibody sandwich (DAS)–ELISA. No cross reactions were observed when RT-PCR products from the two viruses were examined by Southern blot hybridization using BBrMV- and AbaMV-specific digoxigenin-labeled DNA probes. BBrMV was consistently associated with banana bract mosaic disease, as assessed by DAS-ELISA and Southern blot hybridization using DNA probes. The known geographical distribution of BBrMV was extended to include India (Kokkan disease) and Sri Lanka.

Banana (*Musa* spp.) is a vegetatively propagated crop grown both as a staple food and as a major cash crop for local and export markets throughout the tropical and subtropical areas of the world. Three viruses that infect banana have been characterized: banana bunchy top virus (BBTV) (4,27), banana streak badnavirus (BSV) (17), and cucumber mosaic cucumovirus (CMV) (26). A fourth virus-like disease, banana bract mosaic, was first noted in the Philippines in 1979 (18) and, subsequently, yield losses of up to 40% have been recorded (23). The characteristic mosaic symptoms on the flower bracts give the disease its common name. The virus has characteristics typical of a potyvirus. The causal agent is transmitted in the nonpersistent manner by several species of aphids (18). Although efficient purification methods have not been reported, partially purified extracts have been obtained and contain flexuous virus-like particles approximately 750×11 nm (20). The C-terminal half of the coat protein and the 3′-untranslated region of a Philippine isolate of banana bract mosaic virus (BBrMV) have recently been cloned and sequenced, demonstrating it to be a distinct potyvirus (3). The putative coat protein also reacted in immunoblots with polyclonal potyvirus group-specific antibodies (3). No alternative hosts of BBrMV outside *Musa* are known, and the virus has not been transmitted by mechanical inoculation. Banana plants with similar symptoms and also containing flexuous rod-shaped particles have been observed in India, where the disorder is known locally as Kokkan disease (2).

In the Philippines, *Musa textilis* (abaca or Manila hemp) can be infected by abaca mosaic virus (AbaMV), considered to be a strain of sugarcane mosaic potyvirus (9). AbaMV can also be transmitted to edible *Musa* spp., causing somewhat similar symptoms to banana bract mosaic disease (26), raising the question as to whether the two diseases are caused by the same virus.

This paper describes the purification and some properties of BBrMV, its detection by serological and reverse transcription–polymerase chain reaction (RT-PCR) assays, its consistent association with banana bract mosaic disease, and confirms its classification as a potyvirus. The relationship between AbaMV and BBrMV is also examined.

MATERIALS AND METHODS

Virus isolates. Field samples of banana and abaca with typical symptoms of either banana bract mosaic or abaca mosaic disease were collected in the Philippines, India, and Sri Lanka (Table 1) and imported into Australia under Australian Quarantine and Inspection Service license. Quarantine restrictions prevented propagation of the viruses in Australia. Tissue was either used fresh or frozen at –70°C. BBrMV isolate 509 was used for antiserum production and for cloning, sequencing, and probe production. This isolate was obtained from a single plant of cv. Cardaba (ABB/BBB) from Davao, Philippines, and, when indexed, was shown to be free of other detectable viruses including AbaMV (cDNA probe), BBTV, BSV, and CMV (8). Healthy controls were glasshouse-grown plants of Cavendish cv. Williams (AAA) from Queensland and were free of all detectable viruses, as determined by the tests noted above and electron microscopy of sap extracts.

As positive controls in enzyme-linked immunosorbent assays (ELISA), freeze-dried leaf tissues containing the following potyvirus and rymovirus isolates were used (acronyms by Hull et al. [13]; supplier or Department of Primary Industries reference col-

lection number in parentheses): dasheen mosaic virus (DsMV, isolate 455), johnsongrass mosaic virus (JGMV, isolate 340), sugarcane mosaic virus (SCMV, isolate 366), maize dwarf mosaic virus (MDMV, strain A; R. E. Ford), sorghum mosaic virus (SrMV, strain SCMV-H; R. E. Ford), and wheat streak mosaic virus (WSMV; W. G. Langenberg). As a positive control in RT-PCR, fresh tissue of JGMV isolate 535 was used.

Isolates of other banana viruses were also used for serological comparisons: BBTV (isolate 482), BSV (isolate 517), and CMV (isolate 207).

Electron microscopy. Virion preparations were placed on collodion-coated, carbon-stabilized copper grids, negatively stained with 2% (wt/vol) ammonium molybdate (pH 5.8) or 2% (wt/vol) potassium phosphotungstate (pH 7.0), and viewed in an electron microscope. Tobacco mosaic tobamovirus was used as an internal size standard, and the virions were assumed to be 18-nm wide × 300-nm long (31). Tissue was processed for thin section electron microscopy essentially as described by Greber and Gowanlock (10).

Virion purification. All procedures were carried out at 5°C, unless otherwise specified. Relative centrifugal force in the ultracentrifuge was calculated at R_{av}.

Leaf lamina or midrib tissue of BBrMV-509–infected banana was used fresh or after storage at –70°C for up to 12 weeks. The tissue was diced, frozen in liquid nitrogen immediately, and then finely powdered in a Waring blender (Waring, New Hartford, CT) with a metal bowl. The powdered, frozen tissue was thawed in 5 volumes of extraction buffer (EB; 0.5 M borate [pH 6.8] and 0.2%

[vol/vol] 2-mercaptoethanol). The slurry was then strained through four layers of cheesecloth, and the filtrate was clarified by centrifugation at $10,000 \times g$ for 10 min. Triton X-100 was then added to a final concentration of 2.5% (vol/vol) and the mixture stirred for 1 to 1.5 h. The mixture was centrifuged for 2 h at 40,000 rpm ($125,000 \times g$) through a 15-ml cushion of 30% (wt/vol) sucrose in EB, using Beckman Quickseal tubes in a Type 45 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). Pellets were resuspended in 0.25 M borate buffer, pH 6.8 (1 ml per tube), using a Dounce homogenizer, and then stirred for several hours. The virus particles were further purified by equilibrium centrifugation in cesium chloride (0.44 g/ml) at 10°C for 16 h at 32,000 rpm ($126,000 \times g$, Beckman SW 41 Ti rotor [Beckman Instruments, Inc.]) or 38,000 rpm ($137,000 \times g$, Beckman SW 55 Ti rotor [Beckman Instruments, Inc.]). Gradients were fractionated using an Isco fractionator (Isco, Inc., Lincoln, NE) with Fluorinert chase solution (Isco, Inc.). UV-absorbing, virion-containing fractions were diluted with buffer, concentrated by ultracentrifugation for 1 h at 40,000 rpm ($118,000 \times g$) in a Beckman Type 70 Ti rotor (Beckman Instruments, Inc.), and the resulting pellets resuspended in a small volume of 0.05 M borate buffer, pH 6.8, or 0.05 M sodium phosphate buffer, pH 7.0.

The concentration of purified virion preparations was estimated by UV spectrophotometry, using an extinction coefficient of 2.4 mg/ml/cm, as determined for tobacco etch potyvirus (22).

Serology. A rabbit polyclonal antiserum to BBrMV-509 was prepared using a series of three intramuscular injections of purified virions. The first injection on day 0 contained 140 µg of

TABLE 1. Abaca mosaic virus (AbaMV) and banana bract mosaic virus (BBrMV) isolates used in this experimental work

Virus	Origin	Cultivar/genotype	Isolate no.	Sample type (F/A)[a]	Additional viruses detected
BBrMV	India				
	Bangalore	Nendran/AAB	513	F	
	Kannara	Poovan (syn. Mysore)/AAB	556	F	BSV[b]
	Kannara	Chinia/AAB	557	F	
	Kannara	Enikomban (syn. Nendran)/AAB	559	F	
	Kannara	Ney Poovan/AB	560	F	
	Trichur	Nendran	561	F	
	Philippines				
	Los Baños	Unknown	353	F	
	Davao	Cardaba/ABB/BBB	509	F	
	Los Baños	Abaca (*Musa textilis*)	510	A	
	Davao	Cardaba	511	F	
	Los Baños	Unknown	512	F	
	Davao	Cardaba	514-2	F	
	Davao	Cardaba	514-3	F	
	Davao	Cardaba	514-4	F	
	Davao	Mysore/AAB	514-8	F	BSV
	Davao	Mysore	514-9	F	BSV
	Davao	Kalapua/ABB	514-10	F	BSV
	Davao	Kalapua	514-11	F	BSV
	Mindanao	Grand Naine/AAA	515-5	A	
	Mindanao	Butuhan/BB	515-6	A	
	Mindanao	Grand Naine	515-7	A	
	Laguilayan	Lakatan/AA	515-9	F	
	Los Baños	Unknown	516	F	
	Los Baños	Tangong/AA/AAA	533	F	
	Sri Lanka				
	Wagolla	Embul (syn. Mysore)/AAB	552	F	BSV
	Wagolla	Embul	553	F	BBTV[c]
	Wagolla	Embul	554	F	
	Ganaruwa	Embul	555	F	BSV
AbaMV	Philippines				
	Mindanao	Grand Naine	515-1	A	
	Mindanao	Abaca	515-2	A	
	Baganihan	Abaca	515-3	F	
	Mindanao	Grand Naine	515-4	A	
	Mindanao	Grand Naine	515-16	A	
	Los Baños	Unknown	543	Unknown	

[a] Virus isolates are either experimentally aphid-inoculated (A; supplied by L. Magnaye and L. Heradura, Philippines) or field samples (F).
[b] Banana streak badnavirus.
[c] Banana bunchy top virus.

BBrMV in an equal volume of Freund's complete adjuvant. The second and third injections on days 28 (110 µg) and 49 (200 µg) contained an equal volume of Freund's incomplete adjuvant. Blood was collected on days 35, 42, 56, and 62, and serum from the bleed on day 56 was used unless otherwise specified. Immunoglobulin G (IgG) was prepared from the serum by ammonium sulfate precipitation followed by ion-exchange chromatography and was subsequently used to make alkaline phosphatase conjugates (5).

Antisera to the following members of the family *Potyviridae* were also used (suppliers in parentheses): DsMV (D. E. Purcifull); WSMV (W. G. Langenberg); MDMV, strain MDMV-A (R. Toler); SCMV, strain SCMV-D (R. Toler); JGMV, strain SCMV-JG (D. D. Shukla); SrMV, strain SCMV-I (R. Toler); and potyvirus group monoclonal antibody (Agdia, Elkhart, IN).

Serological relationships were investigated by plate-trapped antigen (PTA)–ELISA (19) or double-antibody sandwich (DAS)–ELISA (5). Polystyrene plates (Immulon 3 or 4; Dynatech Laboratories, Inc., Alexandria, VA; or Maxisorb; Nunc, Roskilde, Denmark) were used, and reaction volumes were 100 µl per well.

For PTA-ELISA, plant tissue was macerated in 0.05 M carbonate buffer, pH 9.6, containing 10 mM sodium diethyldithiocarbamate (1 g of fresh tissue or 0.1 g of freeze-dried tissue/50 ml of buffer) and briefly centrifuged before addition to the ELISA plate. Polyclonal detecting antibodies in crude serum were used diluted 1:1,000 to 1:5,000 in an extract of healthy plant sap (1 g of leaf/30 ml of phosphate-buffered saline (PBS)–Tween (0.1 M sodium phosphate [pH 7.4], 0.14 M sodium chloride, and 0.05% [vol/vol] Tween-20). Monoclonal antibodies were diluted to their optimal concentration in PBS-Tween alone. Bound polyclonal or monoclonal antibodies were, in turn, detected with goat anti-rabbit (A3687 [Sigma Chemical Co., St. Louis] diluted 1:30,000 in PBS-Tween) and sheep anti-mouse (DAP [Silenus, Melbourne, Australia] diluted 1:2,000 in PBS-Tween) alkaline phosphatase conjugates, respectively. Sap extract, antibody, and conjugate incubations were for 2 to 3 h at room temperature or overnight at 5°C.

For DAS-ELISA, plates were coated with IgG (3 µg/ml) for 2 to 3 h at room temperature. Plant tissue was macerated in 0.2 M potassium phosphate, pH 7.0, containing 15 mM EDTA, 2% (wt/vol) polyvinyl pyrrolidone, 2% (wt/vol) polyethylene glycol 6,000, and 0.4% (wt/vol) sodium sulfite (1 g of tissue/10 ml of buffer; B. E. L.

Fig. 1. Purified virions of banana bract mosaic virus negatively stained with 2% potassium phosphotungstate. Bar represents 400 nm.

Lockhart, *personal communication*) and briefly centrifuged before addition to the plate and incubation overnight at 5°C. An anti-BBrMV IgG alkaline phosphatase conjugate, diluted 1:500 in PBS-Tween, was next added and incubated for 3 to 4 h at room temperature. For both forms of ELISA, hydrolysis of the substrate *p*-nitrophenyl phosphate (1 mg/ml) was monitored at A_{410} with a Dynatech MR7000 ELISA plate reader (Dynatech Laboratories, Inc.). Samples were considered positive when the mean absorbance of duplicate wells exceeded twice the mean absorbance of appropriate healthy controls.

The sensitivity of the DAS-ELISA was determined using a dilution series of purified BBrMV-509 in a 1:10 diluted extract of healthy banana. Antibody titer was assessed by PTA-ELISA and defined as the maximum dilution giving a positive reaction and an $A_{410} > 0.1$ after 90 min of substrate incubation.

Gel electrophoresis and immunoblotting. Virions of BBrMV-509 were dissociated by boiling for 2 min in 62.5 mM Tris-HCl, pH 6.8, containing 3% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (vol/vol) bromophenol blue. Proteins were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) (15) and visualized by staining with Coomassie blue (24). Size estimates were made by comparison with Bio-Rad low range protein molecular weight markers (Bio-Rad Laboratories, Hercules, CA). Separated proteins were electrophoretically transferred to 0.45-µm nitrocellulose membrane (Bio-Rad Laboratories) using a Bio-Rad mini trans-blot cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Protein transfer was monitored and size estimates determined with prestained molecular weight markers (Rainbow markers; Amersham International plc, Little Chalfront, United Kingdom). After transfer, the membrane was air-dried, rinsed with 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 1 mM EDTA (rinse buffer, RB), and then blocked by incubation in 100 ml of RB containing 5% (wt/vol) skim milk powder for 60 to 90 min at room temperature. Proteins were reacted with anti-BBrMV polyclonal IgG (5 µg/ml) prepared in RB containing 0.1% Triton X-100 (RBT) and an extract of healthy banana sap (1 g/100 ml of RBT). Bound antibodies were detected with goat anti-rabbit IgG-alkaline phosphatase conjugate (A3687) diluted 1:30,000 in RBT. Both detecting antibody and conjugate solutions were used at the rate of 0.125 ml/cm² of membrane and were each agitated with the membrane for 60 min at room temperature in sealed plastic bags. After each incubation, the membranes were washed five times, 5 min each, by vigorous agitation with about 100 ml of RBT. Bound conjugate was detected with the substrates BCIP/NBT (Bio-Rad Laboratories) used according to the manufacturer's instructions.

Nucleic acid extraction and RT-PCR. Two methods were used to prepare RNA template for RT-PCR.

Total nucleic acid extract (TNAE). Total nucleic acids were extracted from plant tissue using a method modified from Pearson et al. (21). Frozen tissue samples were ground with a chilled mortar and pestle at the rate of 1 g per 1 to 2 ml of 50 mM Tris-HCl (pH 7.2) containing 100 mM sodium acetate, 10 mM EDTA, and 0.1% (vol/vol) monothioglycerol. The extract was centrifuged for 2 min at 12,000 × *g* (5°C), and, to 300 µl of supernatant, there was added 30 µl of 10% (wt/vol) SDS and 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was vortexed for 1 min, centrifuged (5 min at 12,000 × *g* at 5°C), and the supernatant reextracted with another 600 µl of phenol/chloroform/isoamyl alcohol. To the final aqueous phase was added 0.05 volume of 3 M sodium acetate, pH 5.5, and 2.5 volumes of ice-cold ethanol. The extract was stored at –70°C for 2 h and then centrifuged, and the pellet was washed with 70% ethanol. The final pellet was dried in vacuo, and then resuspended in 20 µl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) or sterile distilled water including 10 mM dithiothreitol (DTT) and 40 units of RNasin (Progen, Brisbane, Australia).

"Leaf soak" extract. Extracts were made essentially as described by Thomson and Dietzgen (29). Frozen tissue samples (1 to 2 mm³)
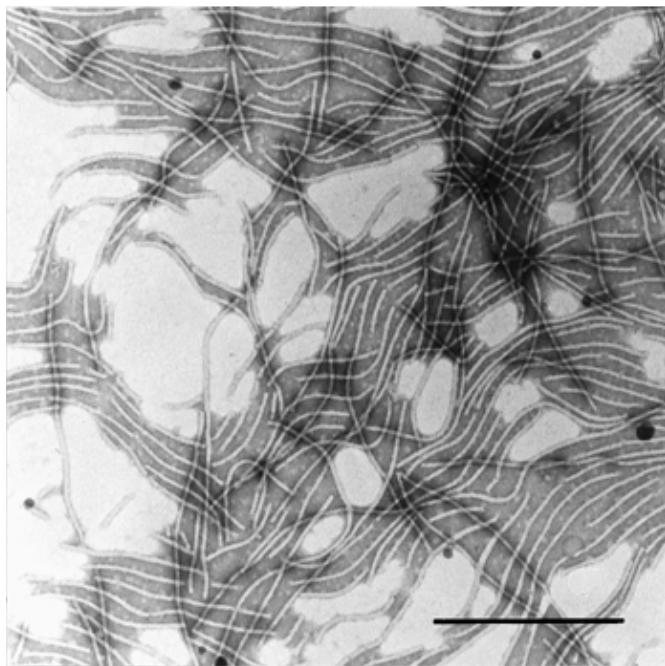
were crushed in 30 µl of 100 mM Tris-HCl, pH 8.4, containing 1.0 M KCl and 10 mM EDTA, and heated at 95°C for 5 to 10 min.

Reverse transcription and PCR were either done separately or combined in a single tube. In the former method, TNAE (0.2 µl) was diluted to 8 µl in diethylpyrocarbonate (DEPC)-treated water, heated to 65°C for 10 min, and then immediately placed on ice. Tube contents were collected by brief centrifugation, and then 5 µl of bulk first-strand cDNA reaction mix including Moloney murine leukemia virus reverse transcriptase (Pharmacia Biotechnology Inc., Uppsala, Sweden), 1 µl of DTT solution (200 pmoles), and 1 µl (20 pmoles) of downstream primer D341 (16) were added and incubated at 37°C for 1 h.

Degenerate primers from the core of the coat protein were used in PCR (16). Reaction volumes of 25 µl contained 2 µl of first-strand cDNA, 5 pmoles each of primers U341 and D341, 2 units of *Taq* polymerase (Gibco BRL, Life Technologies, Gaithersburg, MD), 25 nmoles of each deoxynucleoside triphosphate, 1.5 mM MgCl$_2$, and 2.5 µl of 10× PCR buffer (1× = 20 mM Tris-HCl, pH 8.4, and 50 mM KCl; Gibco BRL, Life Technologies). Tubes were made up to volume with reverse osmosis (RO) water and overlaid with 20 µl of mineral oil. Thirty-three reaction cycles were performed, with periods of 94°C/30 s, 56°C/3 min, and 72°C/1 min (6). All amplifications were carried out in a Hybaid Omnigene thermal cycler (Hybaid, Ltd., Teddington, Middlesex, United Kingdom), and 8 µl of product was analyzed by electrophoresis on agarose gels in 0.5× or 1× Tris-borate-EDTA (TBE), followed by staining with ethidium bromide (24).

For the single-tube RT-PCR, RNA template was either 2 µl of "leaf soak" extract diluted 1:10 in RO water, or 2 µl of undiluted TNAE. The RT-PCR mixture and cycling parameters were as described for PCR, except that, for RT-PCR, the mixture also contained 20 units of reverse transcriptase (Superscript I; Gibco BRL, Life Technologies), and prior to commencing the PCR cycling parameters, there was a 60-min incubation at 37°C, followed by a 90-s denaturation step at 94°C.

The detection limit of the single-tube RT-PCR using a "leaf soak" extract was examined using template dilutions of 1:10 through 1:3,200.

Cloning and sequencing of RT-PCR products. RT-PCR products were cloned using the pCRScript SK(+) cloning kit (Stratagene Inc., La Jolla, CA) according to the manufacturer's instructions. Suspected viral inserts were selected by insert size and confirmed by cycle sequencing using T3 and T7 primers in an Applied Biosystems 373A automated sequencer (Applied Biosystems, Inc., Foster City, CA). The sequences were translated and aligned using the GCG software package (7) and the ClustalW program (28), respectively, both available through the Australian National Genome Information Service at the University of Sydney.

Southern blot hybridizations. Procedures were essentially as described by Sambrook et al. (24). For Southern blot analysis, RT-PCR products were electrophoresed on 0.8% agarose gels in 1× TBE and capillary blotted to Hybond-N nylon membranes (Amersham International plc). Digoxigenin-labeled probes of 341 bp were prepared from cloned inserts of BBrMV-509 and AbaMV-543 using a PCR-labeling kit (Boehringer Mannheim, Mannheim, Germany). Prehybridizations, hybridizations, and stripping of the blots were done essentially according to the manufacturer's instructions, and a chemiluminescent detection system (Lumigen; Boehringer Mannheim) was used. Hybridizations were at 45°C overnight in Easy Hyb buffer (Boehringer Mannheim). Washes were done at high stringency (0.1× SSC/65°C; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) or at moderate stringency (2× SSC/65°C) (12).

RESULTS

Virion purification. In preliminary experiments, a number of potyvirus purification methods were tried (11,20,30), though none resulted in a high yield of relatively pure virions. With the purifi-

cation method developed in this study, purified virion preparations that were essentially free of host contaminants were obtained, as assessed by electron microscopy and SDS-PAGE. The particles were not aggregated (Fig. 1), had an $A_{260/280}$ of approximately 1.17 (uncorrected for light scattering), and banded in a zone in cesium chloride gradients equivalent to a density of 1.29 to 1.31 g cm^{-3}. Yields were in the order of 4 mg/kg of tissue, and the virus was successfully recovered from lamina or midrib tissue from fresh or frozen material.

Electron microscopy. Particles from a purified preparation had a mean length of 725 nm (25 particles measured). BBrMV-infected cells contained pinwheel inclusions and scrolls typical of those associated with infection by a potyvirus (Fig. 2).

BBrMV protein. Three major protein bands with estimated sizes of 31, 37, and 39 kDa (mean of four determinations) were visible on Coomassie-stained SDS-polyacrylamide gels of dissociated BBrMV (Fig. 3A, lane 2). These bands were absent from corresponding healthy preparations (Fig. 3A, lane 3). All three bands reacted specifically in immunoblots with anti-BBrMV polyclonal antibodies that had been cross-absorbed with healthy banana sap (Fig. 3B, lane 1) and were present when preparations were dissociated fresh or after storage at –20°C for several weeks. No antigen was detected in immunoblots from corresponding healthy banana preparations (Fig. 3B, lane 2).

Serology. Serum collected from all four bleeds had a virus-specific titer in PTA-ELISA of 10^{-5}.

BBrMV was detected in tissue extracts from field-infected banana by both PTA- and DAS-ELISA. In PTA-ELISA, cross-absorption of the detecting antibodies with a tissue extract from healthy bananas was essential. For example, after 35 min of substrate incubation, the A_{410} values for a healthy and a diseased extract were 0.04 and 0.32, respectively, when the detecting antibody was cross-absorbed, but 0.12 and 0.38 when not cross-absorbed. All 16 banana bract mosaic disease samples tested from the Philippines, the four samples from Sri Lanka, and the six samples from India reacted positively in DAS-ELISA with the BBrMV antibodies. No reaction was obtained from plants infected with BBTV, CMV, or BSV alone. In a typical DAS-ELISA, after 80 min of substrate incubation, the A_{410} value for a healthy banana sample was 0.01 and, for four infected samples, were 1.16, 0.30, 0.70 (Philippines bract mosaic disease, isolates 509, 511, and 515-5,
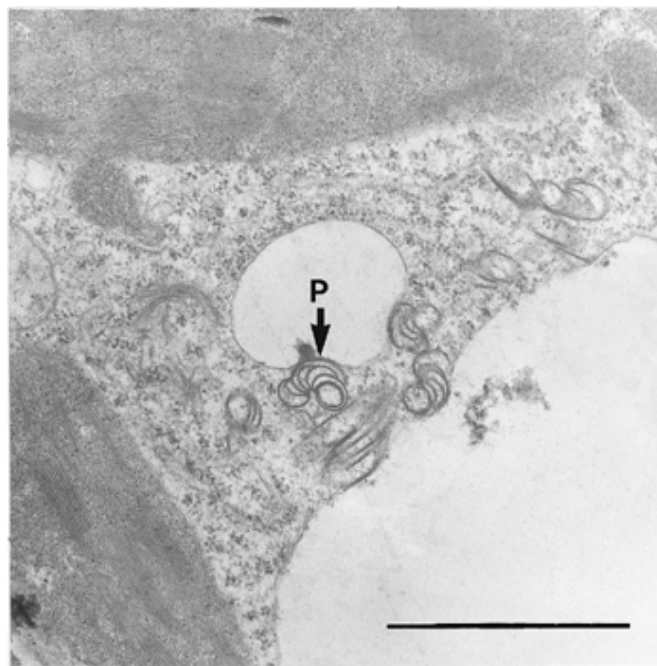

Fig. 2. Pinwheel inclusions (P) in a banana bract mosaic virus-infected cell. Bar represents 1 µm.

respectively), and 0.26 (Indian Kokkan disease, isolate 513). In a second test, healthy samples averaged 0.02, and five infected samples were 0.80 (BBrMV-509), 0.36, 0.57, 0.80, and 0.10 (Sri Lankan samples). For DAS-ELISA, the dilution endpoint of detection of purified BBrMV, prepared in a 1:10 extract of healthy banana sap, was 16 ng/ml.

Serological relationships between BBrMV, AbaMV, and other potyviruses. Positive reactions were obtained in PTA-ELISA between the Agdia potyvirus group monoclonal antibody (Agdia, Inc.) and five of 10 Philippines field isolates of BBrMV. In one test, healthy control samples gave an A_{410} of 0.1, negative test samples (isolates 515-5, 515-7, and two Philippine field samples) were 0.04 to 0.08 and positive samples were 0.29, 0.33, 0.36, and 0.68 (isolates 533, 512, 516, and 515-6, respectively). In a second test, A_{410} values for a healthy control and a BBrMV-509 test sample were 0.02 and 0.07, respectively.

Two isolates each of BBrMV (509 and 516) and AbaMV (515-3 and 543) were tested in PTA-ELISA against a range of potyvirus and rymovirus antisera (Table 2). Weak serological reactions were detected between BBrMV isolates and antisera to SCMV, MDMV, SrMV, DsMV, and WSMV. In reciprocal tests, JGMV, MDMV, SCMV, and SrMV did not react with antiserum to BBrMV (data not shown). Weak serological reactions were detected between AbaMV and antisera to BBrMV and JGMV, while moderate reactions occurred with antisera to SCMV, MDMV, SrMV, and DsMV. In all these tests, strong positive reactions, usually at least 10× the healthy control, were obtained for all homologous virus/antibody combinations.

Three isolates of suspected AbaMV (515-1, 515-2, and 515-3) did not react (A_{410} 0.01) and one (515-4) reacted only weakly (A_{410} 0.074) in DAS-ELISA using antibodies to BBrMV. A fifth isolate (510, originally supplied as AbaMV) reacted strongly (A_{410} 0.97). In the same test, healthy banana sap gave an A_{410} of 0.01 and seven BBrMV-infected samples were in the range A_{410} 0.65 to 1.52. AbaMV samples 515-1 and 515-2 were shown to contain flexuous, rod-shaped particles by electron microscopy, and all samples gave positive PTA-ELISA reactions against antibodies to other potyviruses (data not shown) and an amplification product of the expected size in RT-PCR. Thus, the negative ELISA reac-

tions between AbaMV and BBrMV antibodies are probably not due to the absence of potyvirus virions.

RT-PCR. Using U341/D341 primers in RT-PCR, the expected amplification product of approximately 340 bp was obtained from all 17 BBrMV-infected bananas and six AbaMV-infected bananas and abaca tested, but not from healthy bananas (Figs. 4 and 5).

Using the single-tube RT-PCR with "leaf soak" extract, a similar degree of amplification was obtained with reverse transcriptase concentrations of 20 to 200 units per tube. Lower, but detectable, levels were obtained with 5 units, and no product was detected with 0.5 units. The presence of RNasin in the RT-PCR mix resulted in a marginal increase in the quantity of PCR product. The greatest amount of specific cDNA was amplified with a 1:10 dilution of the "leaf soak" extract, but detectable product was obtained with up to a 1:1,600 dilution. BBrMV was still detectable in a "leaf soak" extract after storage at –20°C for 34 days and two cycles of freeze-thawing.

Using the single-tube RT-PCR with "leaf soak" extract, specific cDNA was amplified from seven of eight BBrMV isolates tested and three of four AbaMV isolates tested. The isolates that were negative in this test were retested using TNAE as template, and specific cDNA amplified in both cases.

Southern blot hybridizations. The BBrMV-specific probe (p509-5) gave a positive reaction with all 17 BBrMV isolates tested under moderate stringency wash conditions and with all except BBrMV-513 under high stringency wash conditions (Fig. 5). No reaction was obtained with any of the six AbaMV isolates nor with any of the four healthy control samples. Conversely, the AbaMV-specific probe (p543-4) reacted under high stringency wash conditions with all six AbaMV isolates, but not with any of the BBrMV isolates or healthy controls (Fig. 5).

Cloning and sequencing. From a single cloning experiment with AbaMV-543, one virus-specific cloned insert of about 340 bp was selected for probe generation.

From three separate cloning experiments, a total of 16 inserts of 341 bp derived from BBrMV-509 field-infected banana were sequenced. For 14 clones, the 92 amino acids translated from the coding sequence between the primer sites were identical to the consensus amino acid sequence of BBrMV (3). Two clones differed by a single amino acid substitution (Fig. 6).

DISCUSSION

The properties of BBrMV are consistent with other members of the genus *Potyvirus*. It is transmitted in the nonpersistent manner
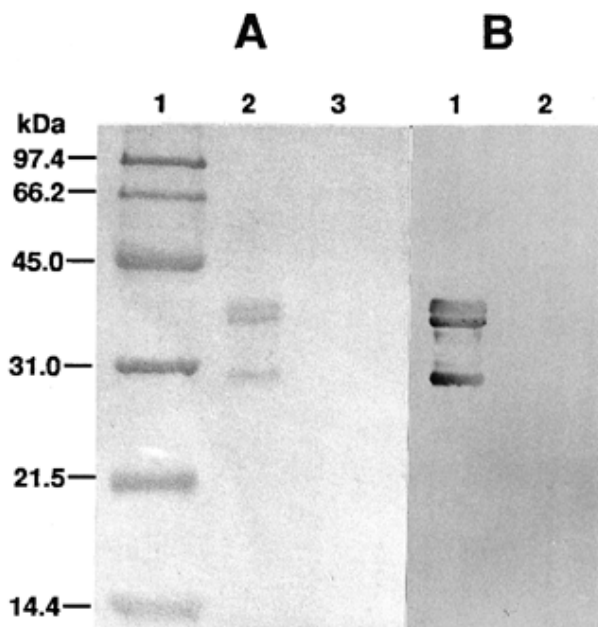

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses of banana bract mosaic virus (BBrMV) proteins. **A**, SDS-PAGE: lane 1, molecular weight markers; lane 2, purified BBrMV; and lane 3, preparation from healthy banana cv. Williams. **B**, Immunoblot: lane 1, purified BBrMV and lane 2, preparation from healthy banana cv. Williams.

TABLE 2. Plate-trapped antigen–enzyme-linked immunosorbent assay reactions between BBrMV[a] and AbaMV and antisera to various members of the family *Potyviridae*

	Virus isolates			
	BBrMV		AbaMV	
Antisera	509	516	515-3	543
BBrMV	14, 19[b]	NT[c]	2.6, 2.9	<2, 2.4
DsMV	NT	2.6, 3.5	5.3	NT
WSMV	NT	3.1, 3.7	NT	NT
JGMV	<2	<2	<2	2.4
MDMV	2.6	<2, <2	17	8.3
SCMV	3.5	<2, 2.1	NT	11
SrMV	2.1	<2, 2.2	5.5	4.3
Agdia MAb	6.3	3.7	11	NT

[a] BBrMV = banana bract mosaic virus, AbaMV = abaca mosaic virus, DsMV = dasheen mosaic virus, WSMV = wheat streak mosaic virus, JGMV = johnsongrass mosaic virus, MDMV = maize dwarf mosaic virus, SCMV = sugarcane mosaic virus, SrMV = sorghum mosaic virus, and Agdia MAb = Agdia potyvirus group monoclonal antibody (Agdia, Inc., Elkhart, IN).

[b] Factor by which A_{410} of test sample exceeds that of appropriate healthy control, in either one or two separate experiments.

[c] NT = not tested.

by several aphid species (8,18,20), has flexuous, rod-shaped particles about 750 × 11 nm (20), and has a coat protein that is related to other potyviruses at the serological and nucleotide sequence levels (3). The current work describes a virion purification method that has allowed antiserum production and physicochemical characterization. The properties reported here have also confirmed the classification of BBrMV as a potyvirus, i.e., presence of pinwheel inclusions in infected cells, virions with a length of about 725 nm, serological cross-reaction in ELISA with other potyviruses, and the amplification of the expected product by RT-PCR with potyvirus degenerate primers. BBrMV from the Philippines appeared only distantly serologically related to AbaMV, DsMV, MDMV, SrMV, SCMV, and WSMV and was serologically unrelated to JGMV. Some, but not all, BBrMV isolates gave positive reactions with the Agdia potyvirus group monoclonal antibody (Agdia, Inc.). This result may reflect a low concentration of virions in the samples or, alternatively, epitope variation among isolates, a phenomenon observed with other potyviruses (14; J. E. Thomas, *unpublished data*).

Three major protein bands from dissociated virions with sizes of 31, 37, and 39 kDa were detected by SDS-PAGE. It is possible that these multiple bands are due to the presence of more than one virus. However, other known viruses of *Musa* were not detected by ELISA or nucleic acid hybridization in this sample, and only potyvirus-like particles were observed when purified extracts were

examined by electron microscopy. When cDNA from this sample was amplified by RT-PCR using degenerate potyvirus group primers and the products cloned and sequenced, all 16 inserts from three independent cloning experiments were identified as BBrMV. By analogy with other potyviruses, these three protein bands are probably forms of the coat protein of BBrMV, and the heterogeneity in size may have arisen from either differential cleavage of
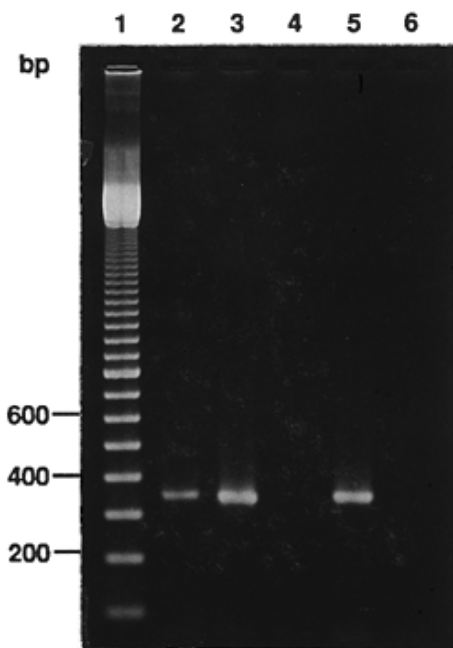

Fig. 4. Agarose gel electrophoresis of reverse transcription-polymerase chain reaction products derived from "leaf soak" extracts. Lane 1, 100-bp ladder; lane 2, banana cv. Cardaba infected with banana bract mosaic virus; lane 3, abaca infected with abaca mosaic virus; lane 4, healthy banana cv. Williams; lane 5, maize cv. Jubilee infected with johnsongrass mosaic virus; and lane 6, healthy maize cv. Jubilee.
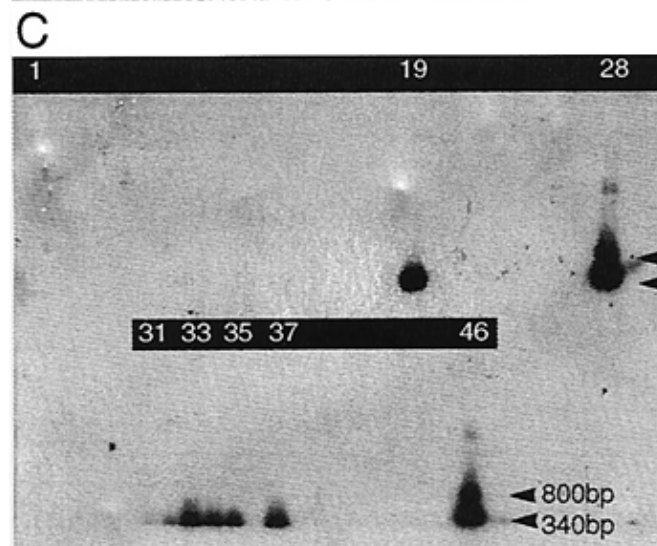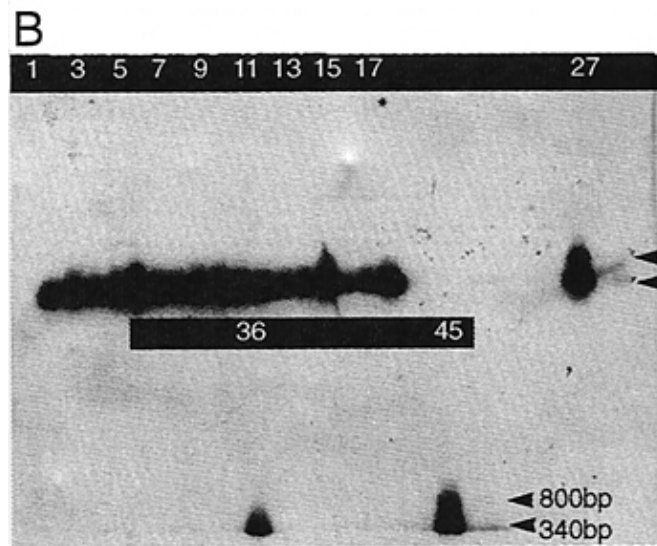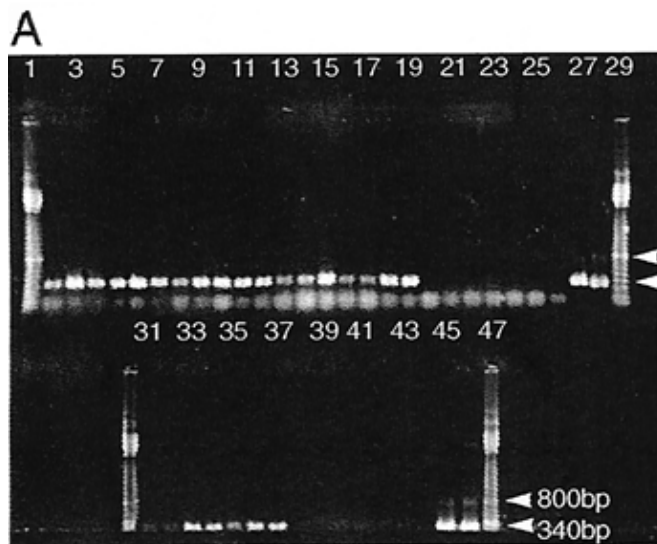
Fig. 5. A, Polymerase chain reaction (PCR) products amplified from total nucleic extracts of samples of banana bract mosaic virus (BBrMV) (lanes 2–18, and 36, including Kokkan disease isolate 513 in lane 16); abaca mosaic virus (AbaMV) (lanes 19, 31–35, and 37); healthy bananas (lanes 20–23 and 38–41); plasmid controls p509-5 (BBrMV, lanes 27 and 45) and p543-4 (AbaMV, lanes 28 and 46); PCR, cDNA, and extraction negative controls (lanes 24–26 and 42–44); and 100-bp ladder size markers (lanes 1, 29, 30, and 47), arrows indicate 340 bp and 800 bp. **B,** Southern blot hybridization of the agarose gel from **A,** reacted with a digoxigenin-labeled BBrMV-specific probe prepared from p509-5 and with chemiluminescent detection. **C,** Same as for **B,** but using an AbaMV-specific probe prepared from p543-4.

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                   10         20         30         40         50         60         70
BBrMV    TSPNLNGTWS MMDKGEQLVY QLKPIIENAQ PTFRQIMAHF SDAAEAYITM RNVTERYMPR WGALRGLNDI
p509-42  ---------- ---------- ---------- ---------- ---------- ---------- G---------
p509-90  ---------- --------L- ---------- ---------- ---------- ---------- ----------


                   80         90
BBrMV    SLARYAFDFY VVTSKTTNRARE
p509-42  ---------- ------------
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Fig. 6. Amino acid alignment of the consensus sequence of part of the core of the coat protein of banana bract mosaic virus (BBrMV) (3), with two clones of BBrMV-509 having amino acid substitutions. Fourteen additional clones of BBrMV-509 were identical to the consensus amino acid sequence.

the polypeptide precursor of the coat protein or from degradation of the coat protein during and after virion purification (25). The latter possibility appears more likely, as Bateson and Dale (3) reported a single major protein of 38 kDa in partially purified preparations of BBrMV.

BBrMV has not been transmitted by any means to hosts outside the genus *Musa* nor to any host by mechanical inoculation (8,20), making the biological purity of isolates difficult to establish and hampering fulfillment of Koch's postulates. Nevertheless, we have provided strong evidence that BBrMV is, in fact, the causal agent of banana bract mosaic disease. BBrMV-509 was obtained from a single field plant. The sample was extensively tested for the presence of other viruses, especially potyviruses, but found to contain BBrMV only. All 16 virus-specific clones obtained from this isolate were identified as BBrMV. BBrMV was consistently associated with the presence of typical disease symptoms in samples from three countries. A cDNA probe prepared from BBrMV-509 reacted specifically with 17 such samples, but not with any of six samples infected with AbaMV. Additionally, a polyclonal antiserum prepared to this isolate reacted strongly with all 24 samples of banana bract mosaic diseased tissue examined in this work, very weakly with AbaMV, and not with samples infected with BBTV, BSV, or CMV.

Symptoms in banana similar to those caused by BBrMV have been noted in India (1,2), where it is locally known as Kokkan disease. Using ELISA, we have shown that bananas with this disease and also banana samples from Sri Lanka were infected with BBrMV. The reaction of an Indian Kokkan disease sample (BBrMV-513) with a BBrMV-509 probe with moderate, but not high, stringency washing is consistent with the observation that they differ by about 13% in nucleotide sequence in this portion of the coat protein gene (C. F. Gambley, A. D. W. Geering, and J. E. Thomas, *unpublished data*).

The detection of BBrMV in field-infected and glasshouse-inoculated banana was achieved by both ELISA and RT-PCR. The minimum concentration of BBrMV detectable by DAS-ELISA was 16 ng/ml, comparable with many other potyvirus ELISA systems, and the assay should prove suitable for routine field screening. The RT-PCR using the degenerate primers U341/D341 has potential as a general potyvirus test for bananas, as a number of isolates of both BBrMV and AbaMV were amplified. The single-tube RT-PCR using the "leaf soak" method was convenient, though not wholly reliable, as one BBrMV isolate and one AbaMV isolate were not detected, even though they tested positive using TNAE as template in RT-PCR. Of all techniques used, the smallest tissue samples were in the "leaf soak" extracts, and sampling errors could allow infection to be missed if the virus is unevenly distributed. Also, the relative sensitivity of RT-PCR with "leaf-soak" extract is probably less than that with TNAE, because of less efficient extraction of virions and possible presence of inhibitors to PCR.

Despite the apparent similarities in symptoms caused by AbaMV and BBrMV in bananas (18,26), the results presented in this work suggest that the two viruses are distinct. Six suspected AbaMV samples gave positive reactions with an AbaMV-specific cDNA probe, but failed to react with a BBrMV-specific cDNA probe and reacted weakly with BBrMV-specific antibodies in ELISA. By contrast, one suspected AbaMV sample (BBrMV-510), originally collected from abaca, did react strongly with the BBrMV-specific probe and antibodies, but not the AbaMV-specific probe, and, thus, may originally have been misidentified. In PTA-ELISA, suspected AbaMV samples reacted relatively more strongly with all antibodies to viruses of the SCMV subgroup tested and more weakly with antibodies to BBrMV than did control BBrMV samples. These results support previous work (9) based on transmission of AbaMV to maize (*Zea mays*) and a reaction between SCMV and antiserum to AbaMV in microprecipitin tests, which suggests that AbaMV may be a member of the SCMV subgroup of potyviruses. Sequencing of the 3′-untranslated region and the coat protein gene of AbaMV is currently underway to clarify this hypothesis.

It appears that AbaMV and BBrMV are distinct potyviruses, but can each infect banana and abaca and, thus, the identification of viruses in both these crops and the indexing of *Musa* germ plasm (1,2) must take both these viruses into account.

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