Evidence for a Double-Stranded Circular DNA Genome in a Second Group of Plant Viruses

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ABSTRACT

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Evidence based on nuclease sensitivity, buoyant density, electron microscopy, and molecular cloning indicates that certain small nonenveloped bacilliform plant viruses may constitute a second group of viruses of higher plants having a circular double-stranded DNA genome. Purified bacilliform virions of *Commelina* yellow mottle virus (CoYMV) contain double-stranded DNA molecules 7.3 kilobase pairs (kbp) in size, 2.1–2.3 μm in length, with a buoyant density of 1.57 g/cm^3 in CsCl-ethidium bromide gradients. DNA extracted from CoYMV virions consists of linear and circular forms, which migrate separately in agarose gels. CoYMV genomic DNA appears to have two single-stranded discontinuities and was not shown to be infectious. Preliminary data on three other viruses similar to CoYMV in size and morphology—banana streak virus (BSV), canna yellow mottle virus (CaYMV), and Kalanchoe top-spotting virus (KaTSV)—indicate that they also contain double-stranded DNA of similar size. These plant viruses are of potential interest for use as vectors for gene transfer in higher plants.

Shepherd et al in 1968 (28) provided the first evidence for a double-stranded DNA (dsDNA) genome in a plant virus, cauliflower mosaic virus (CaMV). Other members of the caulimovirus group subsequently were shown to possess a genome similar to that of CaMV (9). Geminiviruses were shown to constitute a second group of DNA-containing plant viruses, with a single-stranded genome (5). A group of large polyhedral viruses infecting certain unicellular, exsymbiotic, chlorellalike green algae also contain dsDNA (29,30), but to date, the caulimoviruses have remained the sole group of viruses of higher plants having a dsDNA genome.

Small nonenveloped bacilliform viruses have been reported to infect a range of economically important plant species, including cacao (3), rice (25), banana (18), sugarcane (19), canna (34), *Kalanchoe* (8,21), taro (14), yam (6), and *Rubus* (17). Cacao swollen shoot virus (CCSV), rice tungro bacilliform virus (RTBV), banana streak virus (BSV), Kalanchoe top-spotting virus (KaTSV), and the viruses infecting yam and taro cause or are associated with diseases of considerable economic importance, particularly in the tropics and subtropics. Particles of these viruses are bacilliform in shape and approximately 30 × 130 nm in size (3,6,8,14,17,18,21). They differ from plant rhabdoviruses, which also are bacilliform but are larger, have a lipid-containing envelope, and contain single-stranded RNA (26). Rhabdoviruses are larger than the bacilliform bottom component of alfalfa mosaic virus, which measures 18 × 50–60 nm in size and also contains single-stranded RNA (15). None of the small nonenveloped bacilliform viruses has been characterized, no information is available on their genome properties, and they have not been formally assigned to a taxonomic group. In the course of the characterization of CoYMV, a mechanically transmitted virus (22) which occurs naturally in the Caribbean in the common weed *Commelina diffusa* Burm. (24), it was found that purified virions contained DNA rather than RNA (20). This report describes some of the properties of CoYMV DNA and gives preliminary information on apparently similar genomes in BSV, canna yellow mottle virus (CaYMV), and KaTSV.

MATERIALS AND METHODS

Virus purification and nucleic acid extraction. CoYMV was purified from infected leaf tissue of *C. diffusa* by the procedure described for BSV (18) and sugarcane bacilliform virus (SCBV) (19). The virus was further purified by isopycnic banding in Cs_2SO_4. The virus suspension, in 10 mM Tris-HCl, pH 7.4, was adjusted to 40% (w/v) Cs_2SO_4 and centrifuged for 24 hr at 130,000 *g* (max) at 5 C in a Beckman SW28.1 rotor (Beckman Instruments, Inc., Fullerton, CA). The single virus band was collected and freed from Cs_2SO_4 by repeated dialysis against 0.01 M Tris-HCl, pH 7.4.

BSV, CaYMV, and KaTSV were purified from infected leaf tissue of banana, canna (*Canna indica* L. 'The President'), and *Kalanchoe blossfeldiana* Poelln. 'Cinnabar', respectively, by the method described for BSV (18). Further purification by isopycnic banding was not done.

Nucleic acid was extracted from purified virions of CoYMV, BSV, CaYMV, and KaTSV by the method described for CaMV (10,27). Virus suspensions were preincubated with DNase I (25 μg/ml) and RNase A (100 μg/ml) at 37 C for 30 min, followed by digestion with proteinase K (200 μg/ml) in the presence of 0.5% (w/v) sodium dodecyl sulphate (SDS) at 50 C for 30 min. After extraction of the suspension with phenol (once) and chloroform (twice), nucleic acid was precipitated at 0 C by addition of 2 v of absolute ethanol. Precipitated nucleic acid was washed with 70% ethanol and dried under vacuum.

Equilibrium centrifugation of nucleic acid in CsCl-ethidium bromide gradients. Nucleic acid extracted from virions of CoYMV was resuspended in 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) (TE buffer), pH 8.0. The solution was adjusted to a final concentration of 1.55 g/ml CsCl and 600 μg/ml ethidium bromide and centrifuged at 245,000 *g* (max) for 40 hr at 20 C in a Beckman 75 Ti rotor (Beckman Instruments).

Isopycnically banded nucleic acid was collected by syringe and used for buoyant density determination, electrophoretic analysis, and electron microscopy. Before electrophoresis, bound ethidium bromide was removed by butanol extraction and the nucleic acid was reprecipitated with ethanol and resuspended in TE buffer, pH 8.0. Nucleic acid extracted from BSV, CaYMV, and KaTSV was not banded isopycnically before electrophoresis.

Electrophoretic analysis of CoYMV, BSV, CaYMV, and KaTSV nucleic acids. The electrophoretic behavior of nucleic acids

extracted from virions of CoYMV, BSV, CaYMV, and KaTSV was studied in 0.8% agarose gels. The viral nucleic acids were run either untreated or after treatment with enzyme, alkali, or heat. In all cases, agarose gels were prepared in 90 mM Tris-borate, 2 mM EDTA, pH 8.0 (TBE), and the running buffer was TBE containing 0.1% (w/v) of SDS. Electrophoresis was done at 5 V/cm for 2.5–4.5 hr. Nucleic acid bands were visualized by ethidium bromide staining. Lambda DNA/*Hind* III fragments were used as markers. RNase A treatment was done using DNase-free RNase (prepared by heating for 15 min at 100 C) at 100 μg/ml for 1 hr at 37 C. RNase-free RQ1 DNase (Promega, Madison, WI) was used at 25 μg/ml for 30 min at 37 C in a reaction mixture containing 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, and 6 mM $MgCl_2$. S1 nuclease (Bethesda Research Laboratories, Gaithersburg, MD) treatment was done in 30 mM sodium acetate, pH 4.6, 50 mM NaCl, and 1 mM zinc acetate. Denaturation of nucleic acid by alkali and heat-quick-cool were done as described for CaMV (32).

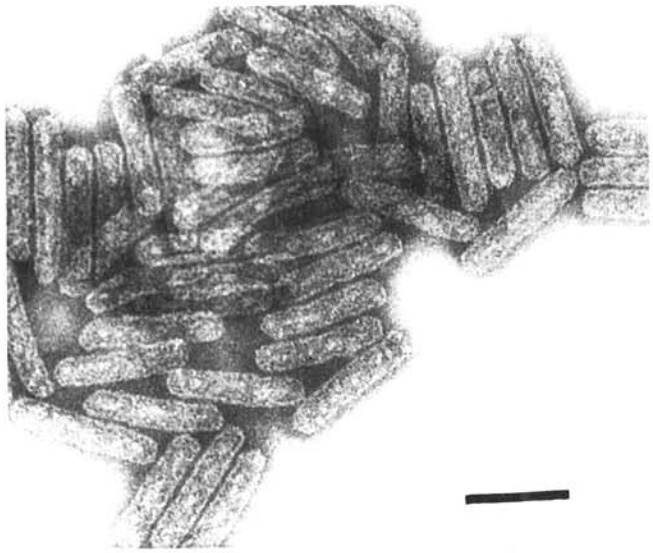


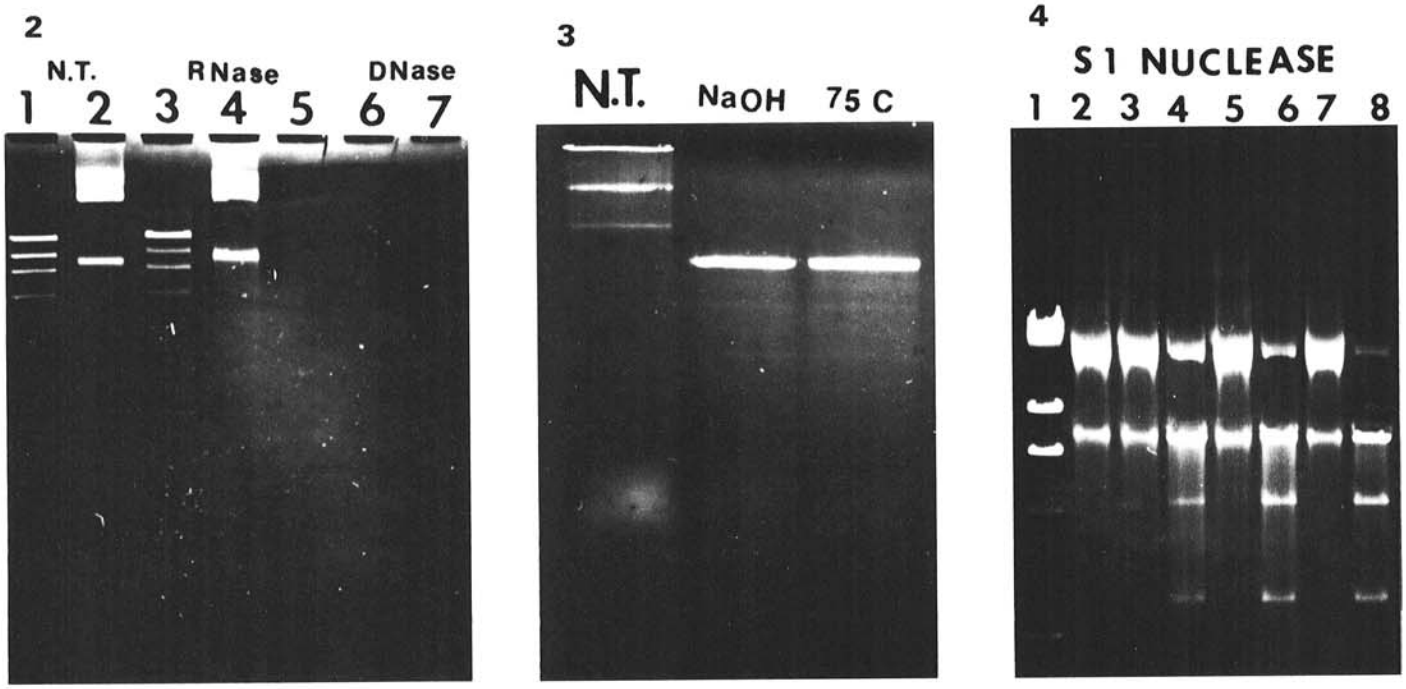
Fig. 1. Virions of *Commelina* yellow mottle virus, purified by isopycnic banding in Cs_2SO_4, negatively stained with 2% sodium phosphotungstate, pH 6.8. Bar = 100 nm.

Electron microscopy. Purified virus was examined after negative staining with 2% sodium phosphotungstate, pH 6.8. DNA was spread for electron microscopy using benzyldimethylalkylammonium chloride (31) and rotary shadowed at low angle with platinum-carbon. Electron microscope magnification was calibrated using stained catalase crystals (33). Contour lengths of DNA molecules were measured from ×4 magnifications of electron micrographs using a Zeiss MOP-3 digitizer (Carl Zeiss, Inc., Thornwood, NY).

RESULTS AND DISCUSSION

Isopycnically banded CoYMV was shown by electron microscopy to be free of contaminating particulate material (Fig. 1). Nucleic acid extracted from purified virions banded at a density corresponding to 1.57 g/cm^3 when centrifuged to equilibrium in CsCl-ethidium bromide gradients.

In nondenaturing agarose gels, most of the native CoYMV nucleic acid migrated as two bands (Figs. 2–4). The faster migrating species which migrates as a single band was designated species I, and the slower migrating species which migrates as a group of bands was designated species II. Native CoYMV nucleic acid was unaffected by RNase treatment but was degraded completely by DNase I (Fig. 2). Heating and rapid cooling, or brief alkali treatment, both of which converted CaMV DNA to



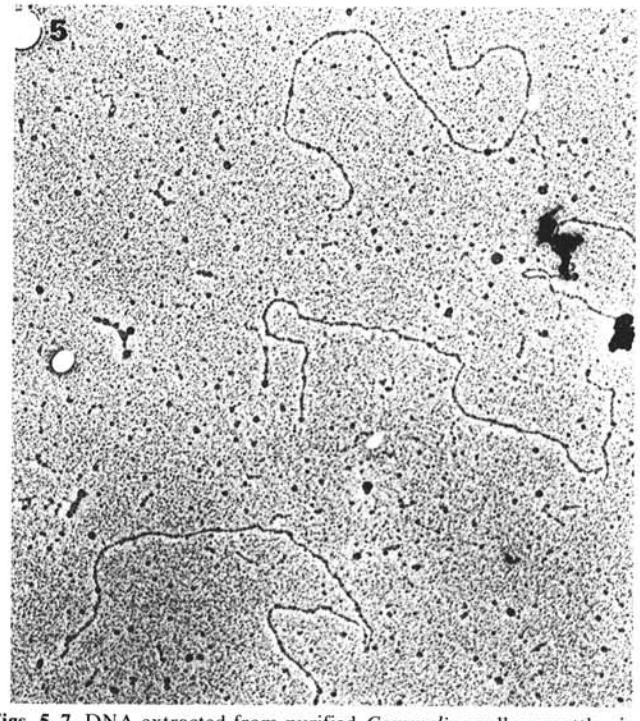
Figs. 2–4. Electrophoretic analysis of untreated and treated *Commelina* yellow mottle virus (CoYMV) DNA. **2,** Lane 1: untreated Lambda DNA/*Hind* III fragments used as markers; lane 2: untreated CoYMV DNA; lane 3: Lambda DNA markers treated with DNase-free RNase A (100 μg/ml for 1 hr at 37 C); lane 4: CoYMV DNA treated with RNase under similar conditions; lane 5: Lambda DNA markers treated with RQ1 DNase (Promega, Madison, WI) at 25 μg/ml for 30 min at 37 C; lane 6: CoYMV DNA treated with RQ1 DNase under similar conditions; lane 7: brome mosaic virus (BMV) RNA treated with RNase. (Untreated BMV RNA not shown.) Gels were 0.8% agarose in 90 mM Tris-borate, 2 mM ethylenediaminetetraacetic acid, pH 8.0 (TBE). Electrophoresis was done in TBE containing 0.1% (w/v) of sodium dodecyl sulphate at 5 V/cm for 3.5 hr. NT = no treatment. **3,** Lane 1: native CoYMV DNA; lane 2: CoYMV DNA denatured by heating for 10 min at 75 C followed by rapid cooling (27); lane 3: CoYMV DNA denatured by 0.25 N NaOH for 1 min at 0 C (27). Electrophoresis was done as described above for 2.5 hr. **4,** Lane 1: Lamba DNA markers; lane 2: untreated CoYMV DNA; lanes 3, 5, and 7: CoYMV DNA incubated at 37 C in the absence of S1 nuclease for 10, 20, and 30 min, respectively; lanes 4, 6, and 8: CoYMV DNA incubated at 37 C in the presence of S1 nuclease for 10, 20, and 30 min, respectively. Electrophoresis was done as described above for 4.5 hr.

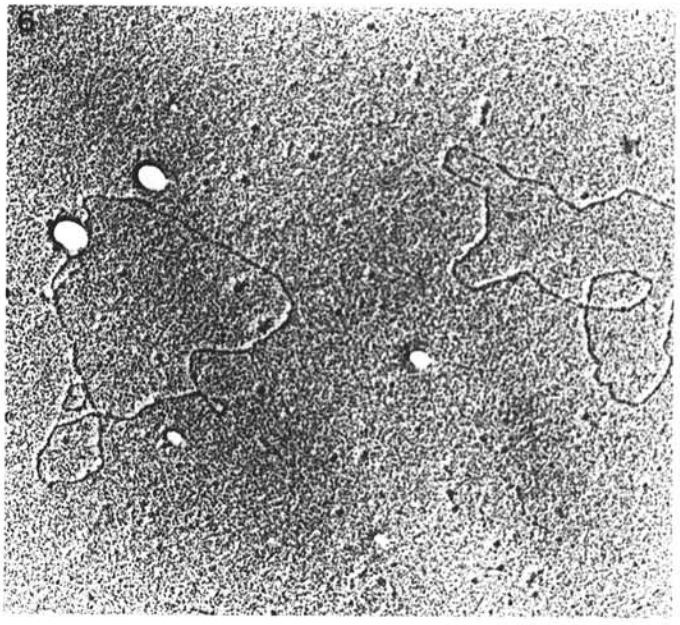
a single-stranded form (32), converted CoYMV nucleic acid to a single band that migrated ahead of band I (Fig. 3). Minor bands migrating ahead of the main band in Figure 3 may possibly arise from linear molecules present in the untreated extracted DNA. Native CoYMV nucleic acid was cut by single-strand-specific S1 nuclease to yield two faster migrating bands (Fig. 4). The DNA species produced by heat or alkali denaturation were almost totally degraded by S1 nuclease (photograph not shown). It was concluded from these results that native CoYMV nucleic acid was a double-stranded DNA molecule containing at least two single-stranded regions or discontinuities.

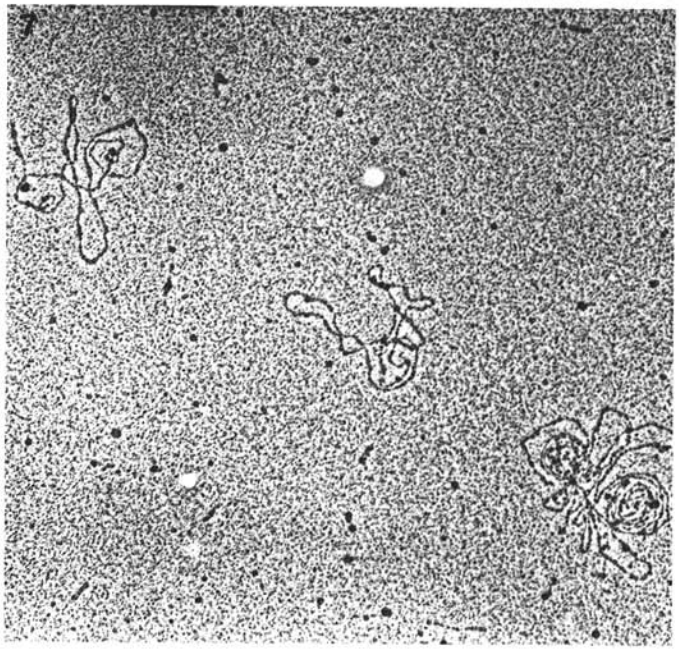
When examined by electron microscopy, unfractionated CoYMV DNA was found to contain a mixture of linear molecules and circular molecules showing varying degrees of twistedness. When electroeluted separately from gels, species I was shown to contain predominantly linear molecules (Fig. 5), whereas species II consisted mainly of circular forms (Figs. 6 and 7). Uninterrupted circular molecules had a contour length of 2.1–2.3 μm (based on 20 measurements). The approximate size of CoYMV DNA, calculated from restriction enzyme digests, was 7.3 kilobase pairs (kbp) (Olszewski and Lockhart, *unpublished*) and was in close agreement with the value estimated from the contour length measurement. The relative proportion of linear species I DNA extracted from CoYMV virions varied from preparation to preparation and was influenced by the kind and degree of manipulation used during DNA extraction and purification. It seems reasonable to postulate that CoYMV DNA occurs in intact virions primarily as a single circular molecule (species II) which becomes disrupted during extraction to yield the linear form.

Nucleic acid was extracted from purified virions of BSV, CaYMV, and KaTSV by the procedure described above for CoYMV. The nucleic acids of these three viruses gave electrophoretic patterns and had molecular weights similar to that of CoYMV, were resistant to RNase, and were degraded by DNase (Fig. 8). These preliminary data suggest that BSV, CaYMV, and KaTSV contain a DNA genome similar to that of CoYMV, and DNA extracted from BSV and CaYMV was found to consist primarily of circular molecules with average contour lengths similar to that of CoYMV (Lockhart, *unpublished*). Restriction digest data indicate that BSV DNA is double stranded and that segments of KaTSV genome have been cloned (Olszewski and Lockhart, *unpublished*). CoYMV is weakly related serologically to RTBV (22), thereby suggesting the possibility that RTBV may contain a DNA genome similar to those of CoYMV, BSV, and KaTSV. Differential histopathological staining also had suggested that the virions of the mimosa bacilliform virus contain DNA rather than RNA (23).

The nature of the CoYMV genome invites comparison between the small nonenveloped bacilliform viruses and caulimoviruses. These viruses resemble each other in being mechanically transmitted (3,9,18,19,21,22), having restricted host ranges (9,18,21,22), and occurring in either random or regular arrays within the cytoplasm but not within nuclei of infected cells (6,8,9,17,24,34). There is no evidence that they occur in discrete cytoplasmic inclusion bodies characteristic of caulimoviruses (9). Caulimovirus







Figs. 5–7. DNA extracted from purified *Commelina* yellow mottle virus (CoYMV). **5,** Linear DNA molecules electroeluted from the faster migrating single band comprising species I CoYMV DNA (see Fig. 2 and text). **6,** Clearly circular and **7,** varyingly twisted molecules electroeluted from the slower migrating set of bands designated as species II CoYMV DNA. Electrophoresis was done in 0.8% agarose gels in 90 mM Tris-borate, 2 mM ethylenediaminetetraacetic acid, pH 8.0 (TBE). DNA was prepared for electron microscopy by the method of Vollenweider et al (31). Magnification was calibrated using stained catalase crystals. Magnification scales in Figures 5, 6, and 7 are ×44,000, ×72,000, and ×58,000, respectively.

genomic DNA (7.6–8.0 kbp) (9) is larger than that of CoYMV (7.3 kbp) but is similar in buoyant density, electrophoretic behavior, and in the occurrence of linear molecules and of circular molecules of varying compactness (9,11). CaMV DNA has two or three S1-sensitive sites (10), whereas CoYMV appears to have only two. The behavior of CoYMV DNA when centrifuged in CsCl-ethidium bromide isopycnic gradients and in agarose gels containing varying concentrations of ethidium bromide (16) (data not shown) supports the conclusion that the DNA of CoYMV, like that of CaMV, is circular but not covalently closed. Blot-hybridization experiments have revealed weak homologies between CoYMV and BSV, and CaYMV and KaTSV, but no similar homology between CoYMV and CaMV DNA (Olszewski, *personal communication*). A further important difference may lie in the infectiousness of the isolated DNA. Extracted CaMV DNA is infectious (13). Purified virions of CoYMV readily infect *C. diffusa* by mechanical inoculation (22), but in two trials purified CoYMV DNA at 50 μg/ml failed to infect *C. diffusa* (Lockhart, *unpublished*). Further studies will be necessary to determine whether the viral genome consists exclusively of circular dsDNA.

Full-length CoYMV DNA has been molecularly cloned, further confirming its postulated double-stranded DNA nature (Olszewski and Lockhart, *unpublished*). The genomes of BSV, CaYMV, and KaTSV also are being characterized. It will be of interest to see what further similarities and differences will be found among the genomic properties of these viruses and caulimoviruses. Recently Hayes et al (7) pointed out that the potential of caulimoviruses as gene vectors in higher plants was limited by the fact that the host range of known caulimoviruses is limited to dicots (9). This drawback is not applicable to the nonenveloped

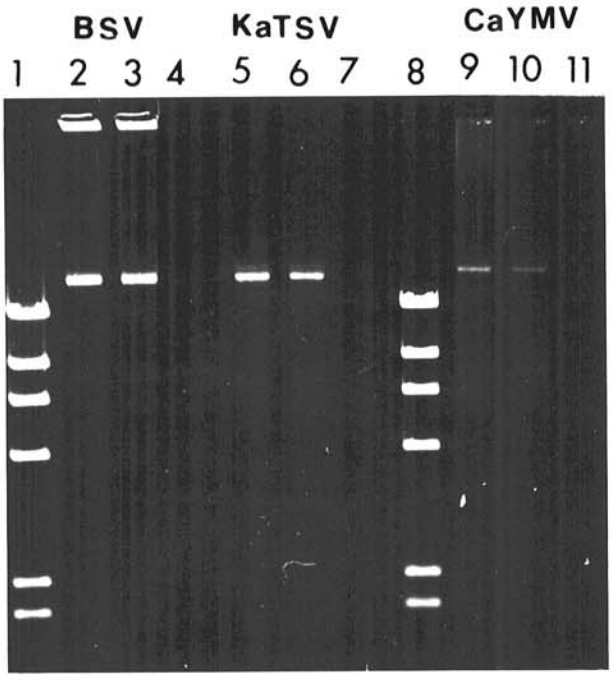


Fig. 8. Effect of nuclease treatment on electrophoretic behavior of nucleic acid extracted from virions of banana streak virus (BSV), Kalanchoe top-spotting virus (KaTSV), and canna yellow mottle virus (CaYMV). Lane 1: untreated Lambda DNA markers; lane 2: untreated BSV nucleic acid; lane 3: RNase-treated BSV nucleic acid; lane 4: DNase-treated BSV nucleic acid; lane 5: untreated CaYMV nucleic acid; lane 6: RNase-treated CaYMV nucleic acid; lane 7: DNase-treated CaYMV nucleic acid; lane 8: RNase-treated Lambda DNA markers; lane 9: untreated KaTSV nucleic acid; lane 10: RNase-treated KaTSV nucleic acid; and lane 11: DNase-treated KaTSV nucleic acid. Viruses were extracted from infected leaf tissue by the method described for cauliflower mosaic virus (10) and purified by differential centrifugation. Nucleic acid extraction and conditions of nuclease treatment are identical to those described for *Commelina* yellow mottle virus. Electrophoresis was done in 0.8% agarose gels in 90 mM Tris-borate, 2 mM ethylenediaminetetraacetic acid, pH 8.0 (TBE), at 5 V/cm for 2.5 hr.

bacilliform viruses, which infect both monocots (CoYMV, BSV, CaYMV, SCBV, RTBV [?]), and dicots (KaTSV, CCSV [?], Rubus yellow net virus (RYNV) [?]). The potential of small nonenveloped bacilliform viruses as vectors would be enhanced if viruses such as cacao swollen shoot virus and rice tungro bacilliform virus prove to have double-stranded DNA genomes. It remains to be seen, however, whether the genomic replicative mechanism of the bacilliform viruses is analogous to that of CaMV and other viruses that use an RNA transcript as a template for replication (4). The presence of S1-sensitive sites in the CoYMV genome is suggestive of this replicative mechanism because the S1-sensitive sites found in the CaMV genome are believed to arise as a result of replication by this mechanism (4). Small bacilliform viruses similar in size to those occurring in plants, but some of which apparently have envelopes, also have been found in invertebrates (1,2,35). Comparison of the properties of these viruses with those of the nonenveloped bacilliform plant viruses would be of interest.

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