Characterization of the genome of cacao swollen shoot virus

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Cacao swollen shoot disease has been known to be caused by a small non-enveloped bacilliform virus for more than 25 years. Purification using a combination of celite filtration, polyethylene glycol concentration and sucrose density gradient centrifugation has yielded concentrated preparations of purified cacao swollen shoot virus (CSSV). Results of nuclease sensitivity tests indicated that the CSSV genome consists of dsDNA which has two single-stranded regions. The approximate size of CSSV DNA calculated from restriction enzyme digests is 7·4 kbp. It is very likely that CSSV is a member of the commelina yellow mottle virus group.

Cacao swollen shoot disease is caused by a mealybug-transmitted virus that occurs in all the main cacao-growing areas of West Africa. It induces swellings on shoots and roots, mosaic and chlorosis and has caused very serious crop losses in Ghana, Nigeria, and more recently, Togo (Partiot et al., 1978). Biological work was done in the 1950s and 1970s on the host range, host–vector relationships, epidemiology of the disease agent and cross-protection against it (for references see Brunt, 1970). The principal means of control in Ghana and Togo has been eradication but several other measures may be used which include the use of less susceptible cultivars (for review see Ollenu et al., 1989).

Progress on the characterization of cacao swollen shoot virus (CSSV) has been hampered by difficulties in purifying it. Brunt & Kenten (1963) obtained partially purified preparations from cacao and described CSSV as the first example of a small non-enveloped bacilliform virus 28 × 130 nm in size. The purification procedure was improved by Adomako et al. (1983) who obtained an antiserum usable for detection by ELISA. Numerous viruses similar in size and morphology have been found in species of economic and alimentary importance such as rice, banana, sugarcane, taro and yam, as well as in some horticultural crops and weeds. Lockhart (1990) reported that commelina yellow mottle virus (CoYMV) contains a circular dsDNA genome of 7·3 kbp and presented preliminary data showing that three other small bacilliform viruses had the same type of genome. In the present paper, we report efforts to obtain more concentrated purified preparations and to determine the nature of the CSSV genome.

Amelonado cacao beans were inoculated at the IRCC Station in Kpalimé with the virulent strain Agou1, similar to the New Juaben strain, using viruliferous Planococcus citri. The young seedlings were maintained in a screenhouse and were checked by ELISA, using an antiserum prepared against strain Agou1, 8 to 10 weeks after inoculation to select plants containing high virus concentrations. The virus was then purified from 30 g batches of infected leaves essentially as described by Adomako et al. (1983), i.e. using pectinase digestion of the extracts, concentration with polyethylene glycol (PEG) 6000 and filtration through celite to remove most of the mucilage and host contaminants. CSSV-containing fractions (50 and 30 g/l PEG in the elution buffer) were pooled and, after addition of sodium azide, sent by airmail to the Virology Laboratory in Montfavet for purification. Two to four preparations were sent together because preliminary assays showed that the virus particles were stable when stored for 1 to 2 weeks at room temperature. After two cycles of clarification (10 min at 7000 g) and ultracentrifugation (2·5 h at 62000 g), virus particles were suspended in 0·02 M-citrate pH 7·0, layered on a 10 to 30% sucrose gradient and centrifuged in a Beckman SW41 rotor for 1 h at 35000 r.p.m. Fractions from a large u.v.-absorbing zone in the middle of the gradient were pooled and purified virus was collected from them by ultracentrifugation (Fig. 1).

Nucleic acid was extracted by treating particles with proteinase K (50 µg/ml in 10 mM-Tris–HCl pH 8·0, 5 mM-EDTA) in the presence of 0·5% SDS at 65 °C for 1 h. The suspension was extracted three times with phenol–chloroform (1:1, v/v), once with chloroform, and the nucleic acid was precipitated by the addition of 2·5 volumes of ethanol in the presence of 0·03 M-sodium acetate. The precipitated nucleic acid was washed with 70% ethanol and then absolute ethanol, and suspended in
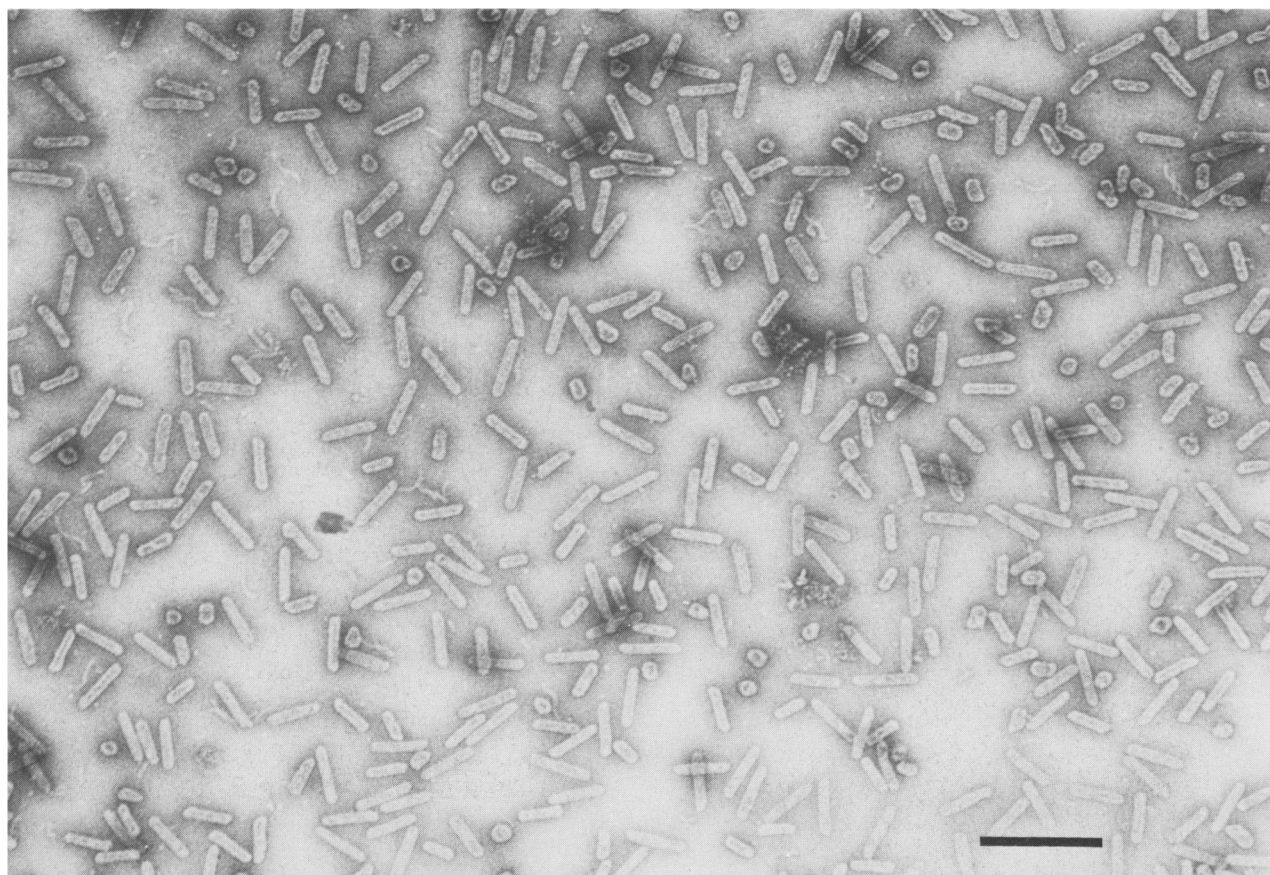
Fig. 1. Purified preparation of CSSV obtained after sucrose density gradient centrifugation. Bar represents 200 nm.

sterile distilled water. Cauliflower mosaic virus DNA (CaMV) (cabbage B strain, PV 147 from ATCC) was a gift from Dr P. Yot.

When electrophoresed in 1% non-denaturing agarose gel in Tris–acetate–EDTA buffer (TAE; 40 mM-Tris, 8 mM-sodium acetate, 1 mM-EDTA, 0·13% acetic acid), CSSV nucleic acid migrated as two main bands and had an electrophoretic pattern similar to that of CaMV DNA (Fig. 2, lanes 2 and 3). Like CaMV DNA, CSSV nucleic acid was not affected by RNase A treatment (Fig. 2, lanes 4 and 5) under conditions in which cucumber mosaic virus RNAs were digested completely (result not shown) (60 units/ml in H_2O for 30 min at 37 °C). On the other hand, CSSV nucleic acid was degraded completely by DNase I (20 μg/ml in 50 mM-sodium acetate, 10 mM-$MgCl_2$, 2 mM-$CaCl_2$ for 30 min at 37 °C) (Fig. 2, lane 7).

Various restriction enzymes were tested for their capacity to cleave CSSV DNA. As shown in Fig. 3, CSSV DNA was efficiently cut, yielding one or more major components and minor ones. A total molecular size of 7·4 kbp was determined by adding the sizes of the three major fragments obtained after digestion with either *Bam*HI or *Eco*RI; this length fits well with that of

the fragments obtained after digestion with *Pst*I or *Sma*I which each seem to have a unique site within the DNA. These experiments demonstrate that CSSV nucleic acid is a dsDNA.

CaMV and CSSV DNAs were denatured by 0·5 M-NaOH for 1 min at 0 °C (Volovitch *et al.*, 1978), which converts dsDNA to ssDNA. CaMV DNA yielded the three expected bands, whereas CSSV DNA appeared as a single component (Fig. 4, lanes 4 and 5). Digestion by S1 nuclease (5 units in 50 mM-sodium acetate pH 4·5, 100 mM-NaCl, 0·3 mM-zinc acetate for 45 min at 37 °C) gave rise to two components migrating ahead of the faster component of the untreated DNA (Fig. 4, lane 7). Although the digestions shown in this figure were incomplete, the same results were obtained when S1 nuclease digestions were complete as judged by the pattern of the treated CaMV DNA (result not shown). These data provide evidence for two single-stranded regions or discontinuities in CSSV DNA, probably one on each strand.

We believe that CSSV DNA occurs in a circular form based on its similarity in behaviour to CaMV DNA. If such a hypothesis is correct, the slower migrating band of
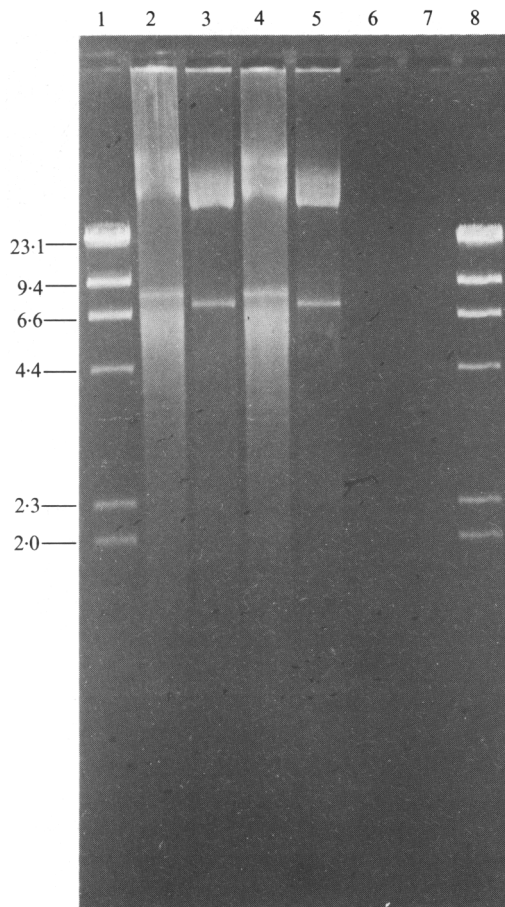
Fig. 2. Agarose gel electrophoresis of untreated or treated nucleic acids. Lanes 1 and 8: λ DNA *Hin*dIII fragments used as molecular size markers. Lanes 2, 4 and 6: untreated, RNase-treated and DNase-treated CaMV DNA, respectively. Lanes 3, 5 and 7: untreated, RNase-treated and DNase-treated CSSV nucleic acid, respectively. Numbers indicate marker DNA sizes in kbp.

Fig. 3. Agarose gel electrophoresis of untreated or treated nucleic acids. Lane 1: λ DNA *Hin*dIII fragments. Lane 2: untreated CSSV DNA. Lanes 3 to 9: CSSV DNA digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dII, *Hin*dIII, *Pst*I and *Sma*I, respectively. Lane 10: φX174 DNA *Hae*III fragments. Lane 11: λ DNA *Hin*dIII + *Eco*RI fragments. Numbers indicate marker DNA sizes in kbp.

the untreated DNA should represent the circular form, whereas the faster migrating band should correspond to the linear molecules arising during DNA extraction (Hull, 1984). Moreover the minor components observed after digestion with the various restriction enzymes might represent the digestion products of the linear forms. Their low abundance precluded determination of their total number and, consequently, the sum of their lengths. Nevertheless, the presence of another DNA population sharing a different restriction map cannot be ruled out.

In order to be sure that the DNA we have characterized was not a host DNA copurified with virions, its presence was checked in total DNA extracts prepared from healthy or infected plants. Two grams of fresh leaves was ground to a fine powder in liquid nitrogen in the presence of 2 g of polyvinylpyrrolidone (PVP K25).
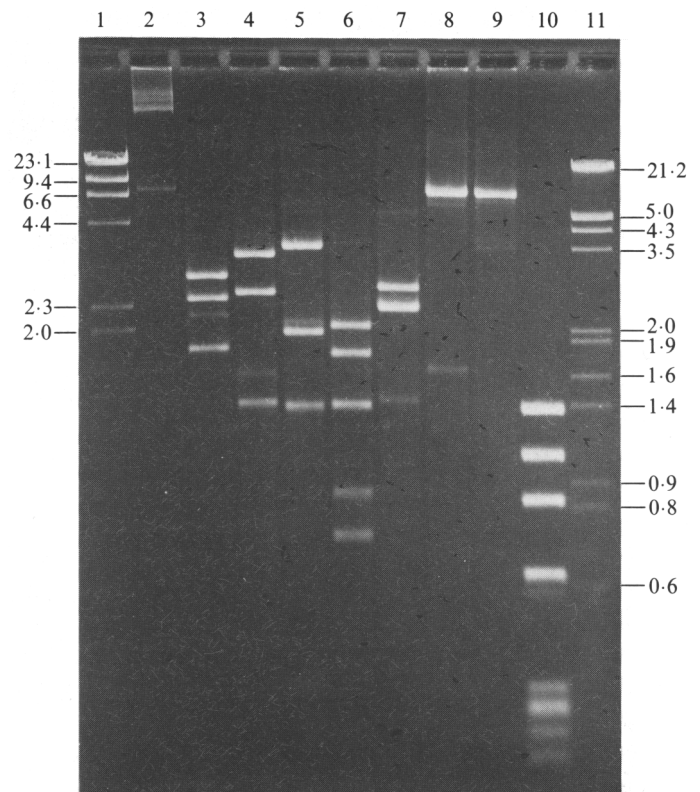
The powder was transferred to a tube containing 10 ml of extraction buffer (0·5 M-tri-sodium citrate, 2% 2-mercaptoethanol, 1% Sarkosyl; pH 8·0) kept at 65 °C. SDS was added to a final concentration of 2%. Cells were lysed at 65 °C for 15 min, then one-half volume of 0·1 M-Tris–HCl pH 8·0-saturated phenol was added. After further incubation for 10 min and centrifugation for 10 min at 10000 *g*, the aqueous phase was extracted with one volume of phenol–chloroform (1:1, v/v). Nucleic acids were precipitated with isopropanol (0·6 volumes) in 2 M-ammonium acetate and resuspended in water (1 ml). RNA and proteins were removed by successive treatments with RNase A and proteinase K as described by Menissier *et al.* (1982). Various amounts of total DNA were loaded onto a 1% agarose gel, electrophoresed for 6 h at 5 V/cm in TAE, and blotted onto nitrocellulose (GeneScreen Plus). The 1450 bp *Eco*RI fragment (Fig. 3, lane 4), cloned in the pUC13 vector, was used as probe. Labelling by using the large fragment of DNA polymerase I with random oligonucleotides as primers, prehybridization and hybridization were carried out as described
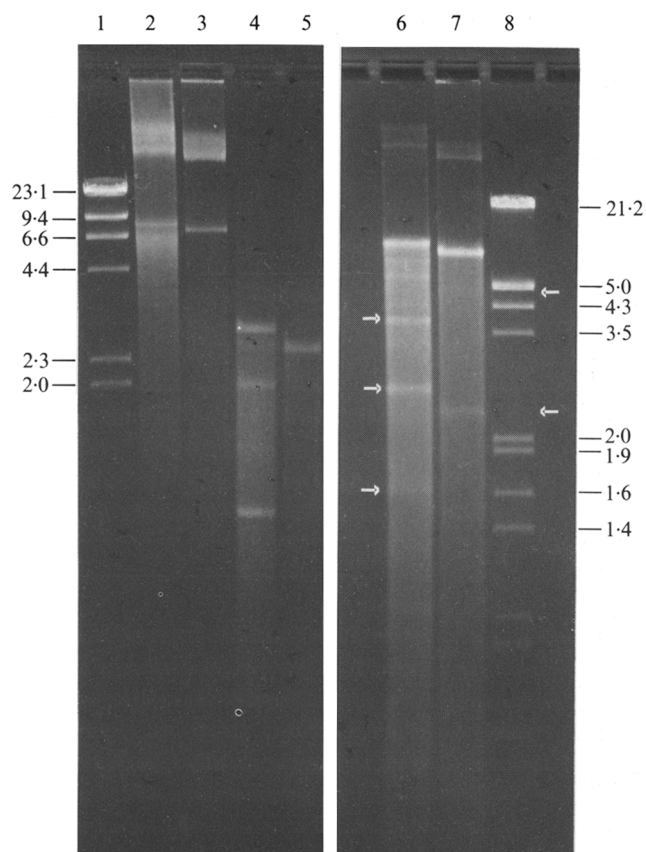
Fig. 4. Agarose gel electrophoresis of untreated or treated nucleic acids. Lane 1: λ DNA *Hind*III fragments. Lanes 2, 4 and 6: untreated, NaOH-treated and S1 nuclease-treated CaMV DNA, respectively. Lanes 3, 5 and 7: untreated, NaOH-treated and S1 nuclease-treated CSSV DNA, respectively. Products resulting from a complete digestion by S1 nuclease are indicated by arrows. Lane 8: λ DNA *Hind*III + *Eco*RI fragments. Sizes (kbp) of M_r markers are indicated alongside.

Fig. 5. Southern blot analysis of total DNA extracted from healthy or CSSV-infected cacao leaves. The gels were loaded with 1 µg (a) or 5 µg (b) of total DNA. Lanes 1: total DNA from healthy leaves. Lanes 2: total DNA from infected leaves. Lanes 3: DNA extracted from purified virions. Lanes 4: total DNA from healthy leaves plus DNA extracted from purified virions. The positions and sizes (kbp) of λ DNA *Hind*III fragments are indicated on the left.

by Feinberg & Vogelstein (1983). When the gel was stained with ethidium bromide before blotting, a single prominent component, corresponding to genomic DNA, was observed (not shown). After hybridization, the two expected components were detected in lanes where DNA purified from virus particles had been loaded (Fig. 5, lanes 3). No hybridization was detected with total DNA from healthy cacao leaves (Fig. 5, lanes 1), even after a 5 day exposure at −70 °C with two amplifying screens. The two viral components were present in total DNA from infected leaves but their migration rates were reduced, particularly when greater amounts of DNA were loaded (Fig. 5, lanes 2). This reduction seemed to be caused by the abundance of mucilage in the extracts since it was also observed when increasing quantities of total cellular DNA from healthy leaves were added to viral DNA (Fig. 5, lanes 4). Moreover, the relative
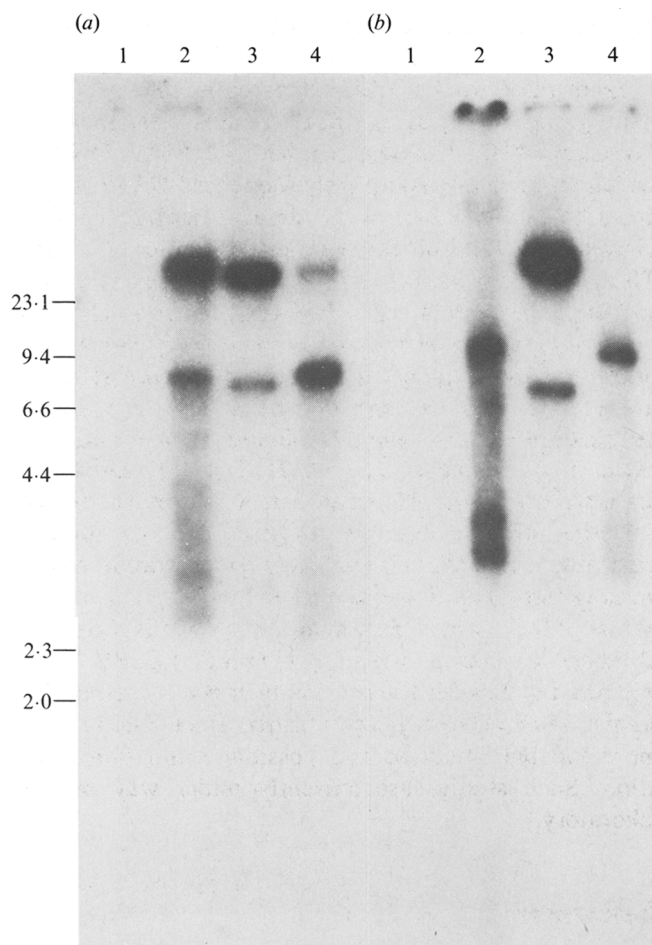
proportions of the two viral forms differed according to the quantity of total DNA from healthy leaves added, leading to the near disappearance of the putative circular form when the greatest amount of DNA was used. Three other components that migrated faster were also detected in total DNA from infected leaves. A similar pattern has been described by Menissier et al. (1982) for total DNA from CaMV-infected turnip leaves. These authors had proposed that the first and third components (according to their migration rates) were incomplete linear forms, and they showed that the second one was a non-encapsidated supercoiled form of the viral DNA. We have no experimental evidence that the forms we have observed correspond to the forms described for CaMV DNA, but the similarity of patterns obtained in Southern

blot experiments is further evidence for the similarity between the two viral DNAs.

From these results, CSSV apparently belongs to the new group of dsDNA plant viruses described by Lockhart (1990). The type member is CoYMV, DNA of which was recently completely sequenced (Medberry *et al.*, 1990). Difficulties in obtaining sufficient purified virus and DNA limit more extensive studies of CSSV. Thus, data concerning the capsid structure are still lacking and examination of DNA molecules by electron microscopy has to be carried out to determine the forms of the various components observed in agarose gels. We are now taking advantage of some improvement in the purification yield by using *Theobroma bicolor* instead of cacao as the propagation host (H. Lot & E. Djiekpor, unpublished result). Nevertheless, improvement in the detection of the virus is hindered by the uneven distribution and the relatively low concentration of the virus in the cocoa tissue as well as by the mucilaginous nature of the extracts (Sageman *et al.*, 1985; H. Lot & E. Djiekpor, unpublished results). Cloning of CSSV DNA will provide, besides the possibility of precise molecular studies, the tools to develop hybridization techniques for more reliable detection and possibly strain differentiation. Such studies are currently under way in our laboratory.

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