Infection of *Theobroma cacao* Using Cloned DNA of Cacao Swollen Shoot Virus and Particle Bombardment

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We thank D. Clement for providing the cocoa pods, B. Delecolle for help with the electron microscopy, J. Rougier for the photography, and J. M. Thresh for his critical review of the manuscript. L. S. Hagen is supported by Centre de Coopération International en Recherche Agronomique pour le Développement-Cultures Pérennes (CIRAD-CP).

Accepted for publication 25 April 1994.

ABSTRACT

Hagen, L. S., Lot, H., Godon, C., Tepfer, M., and Jacquemond, M. 1994. Infection of *Theobroma cacao* using cloned DNA of cacao swollen shoot virus and particle bombardment. Phytopathology 84:1239-1243.

A full-length, monomeric genomic clone of cacao swollen shoot badnavirus was infectious after particle bombardment of cacao seeds from which the testae had been removed. The infected plants showed symptoms typical of the severe form of cacao swollen shoot disease. Progeny virus from plants infected by bombardment reacted in enzyme-linked immunosorbent assay and dot blot hybridization and had small bacilliform particle morphology as observed by immunosorbent electron microscopy. Furthermore, the complex of symptoms that characterize cacao swollen shoot disease can be provoked by a single etiologic agent rather than by a mixture of different viruses or strains. The infectious clone offers new possibilities for the study of swollen shoot disease.

Additional keywords: biolistics, inoculation, microprojectile bombardment.

Cacao swollen shoot badnavirus (CSSV), like other members of the group (21), has small, nonenveloped, bacilliform particles (5) and a double-stranded DNA genome (23). The virus is transmitted by several mealybug species in a semipersistent manner (30). Mechanical transmission is not widely used, because purified virion preparations are infectious only to seeds and not to plants and such preparations are produced with difficulty (4,5). Other plant virus groups with DNA genomes are the caulimoviruses and the geminiviruses.

The ability to experimentally inoculate plants with cloned viral nucleic acid is essential to the study of the molecular genetics of these viruses. In general, when unencapsidated viral DNA (e.g., pronase-treated virus) has been successfully transmitted by mechanical inoculation, success has followed with the cloned DNA. Cloned cauliflower mosaic caulimovirus (CaMV) DNA was shown to be infectious by mechanical inoculation when excised from vector DNA (16,19). Mechanical inoculation with cloned DNA of both components of tomato golden mosaic virus (TGMV) (14) or African cassava mosaic virus (ACMV) (34) is also possible.

Agroinoculation (12) is the most widely employed method to transfer cloned viral material into plant cells. It has been used for CaMV, TGMV, and ACMV and also for viruses whose DNA (viral or cloned) cannot be mechanically transmitted (12). Three other cloned and sequenced badnavirus DNAs, rice tungro bacilliform virus (RTBV), *Commelina* yellow mosaic virus (CoYMV), and sugarcane bacilliform virus, established infections after *Agrobacterium*-mediated inoculation (3,7,25); but for badnaviruses, the clones necessary for agroinoculation must be longer than genome length and can be unstable (3,7,25; L. S. Hagen and M. Jacquemond, *unpublished results*).

A recently developed technique to infect plants with cloned DNA is microprojectile bombardment (also called biolistics or particle acceleration) (33). It was first used successfully with bean golden mosaic geminivirus clones by Gilbertson et al (10), who inoculated the radicles of germinating seeds. Pepper plants at the four-leaf stage have been inoculated with cloned pepper huasteco geminivirus (9). While bombardment worked well for these bipartite geminiviruses in dicotyledonous hosts, it was not as effective in other models. Dasgupta et al (7) bombarded rice seedlings with cloned RTBV, and one of 200 inoculated plants was infected. It is not known whether this limited success was the result of the monocotyledonous plant or the badnavirus used. Later, Chen and Dale (6) bombarded dissected wheat seeds with a cloned dimer of the monopartite wheat dwarf geminivirus and infected four seeds out of 147 inoculated.

Cacao swollen shoot disease provokes diverse symptoms in *Theobroma cacao* L. during the early acute and later chronic stages of infection; these symptoms differ with the cultivar and environmental conditions (2,22). Both severely and mildly virulent isolates are found in different geographical regions (27). The severe form of the disease caused by the Ghanaian strain 1A (New Juaben), which is very similar to that caused by the severe Togo isolate Agou 1 (26), was described by Posnette (27). Other CSSV isolates may provoke only one or more of these symptoms and/or altogether different patterns of mosaic (17,27). As a result of this great variation, it has been suggested that the disease might be the result of a complex of viruses (29).

We previously reported the nucleotide sequence of a full-length clone of CSSV (13), and here we demonstrate its pathogenicity using microprojectile bombardment of cocoa seeds.

MATERIALS AND METHODS

Restriction enzymes, nylon membrane, DNA-labeling reagents, and a chemiluminescent detection kit were obtained from Boehringer Mannheim, Meylan, France. Chemical reagents were supplied by Sigma, St. Quentin Fallavier, France, or Merck, Nogent-sur-Marne, France. Unless otherwise stated, standard molecular methods were used (32).

Cloning and preparation of viral DNA. The plasmid S-2 bearing a full-length copy of the viral DNA cloned into the *Sma*I site of pBluescript has been previously described (13) and was originally derived from purified virus of the severe strain Agou 1 from Kpalime, Togo (23). The viral DNA was recloned by using the unique *Pst*I site to obtain a clone with "sticky ends" when excised from the vector. Plasmid S-2 DNA was restricted with *Sma*I and *Pst*I, and the viral DNA fragments were eluted and recloned into the *Pst*I site of pBluescript to make the plasmid 2P-14. Plasmid DNA was prepared by the alkaline lysis method, extracted with phenol and chloroform, and precipitated in ethanol. The concentration of plasmid DNA was estimated by gel electrophoresis. Plasmid DNA was restricted and reprecipitated with ethanol. The plasmids 2P-14 or S-2, either unrestricted or restricted at their respective cloning sites, were used for mechanical and bombardment experiments as indicated.

Plant growth. Cacao pods, cultivar Amelonado (IFC_5), were sent from the Institut de Recherche du Café et du Cacao, Abidjan, Ivory Coast, and used within 1 wk. On the day of treatment, the seeds were taken from the pod, and the testae were removed. Seeds were kept in plastic boxes lined with water-soaked paper towels before and after treatment and were planted within 3 days. Seeds were germinated in vermiculite, and seedlings were grown in potting soil in a growth chamber regulated at 22 C with a 12-h day-night cycle and watered with nutrient solution. Standing water was kept below the growth racks to maintain 70–80% humidity. The plants were scored for symptoms every 10 days for 18 wk.

Mechanical inoculation. Whole seeds were lightly dusted with Carborundum, and 10 μl of inoculum was gently rubbed over the entire surface of the bean until all liquid was absorbed. Two groups of 48 seeds were inoculated with 5 and 10 μg of 2P-14 per milliliter restricted with *Pst*I. Seventeen controls were inoculated with water. Plants were observed for 9 mo, and 12 randomly chosen plants were tested with a dot blot hybridization test.

Particle bombardment. The bombardment apparatus and conditions were as described by Godon et al (11). For each series of five shots, DNA was precipitated on tungsten particles by the successive addition, while vortexing, of 1 μl of DNA (at 5 μg/μl), five successive 1-μl amounts of 95% ethanol, and then 11 μl of sonicated tungsten particles kept in suspension in cold 95% ethanol (100 μg/μl). Three microliters of this mixture was placed immediately on each aluminum rupture disk and used for bombardment after the particles had dried. Two prepared whole cocoa seeds were held closely together on double-sided tape in the center of a plastic petri dish and bombarded simultaneously. The petri dish was placed on a solid support (in experiment I) or on a foam, resilient support (in experiment II) at a target distance of 7 cm. Bombardment was done with a 25-ms pulse of 650-kPa helium gas in a vacuum of 20 kPa.

ELISA and dot blot hybridization tests. Extraction of leaf tissue with symptoms and enzyme-linked immunosorbent assay (ELISA) were performed essentially as described by Sagemann et al (31). Immunoglobulin G and conjugate, used at a 1/500 dilution, were purified from antisera raised against purified virus of severe CSSV isolate Agou 1 from Kpalime, Togo (H. Lot and E. Djiekpor, *unpublished results*). ELISA was done on samples from all plants at 8 wk in experiment I and at 14 wk in experiment II. Dot blot hybridization was done along with the ELISA test for all plants in experiment I and for a sampling of plants of each group in experiment II and the mechanical inoculation experiment.

Total nucleic acid was purified by a slightly modified method of Manning (24). All steps were done in microcentrifuge tubes. About 20 mg of leaf tissue was frozen in liquid nitrogen and ground in the microcentrifuge tube with a fitted plastic pestle. Seven hundred microliters of Tris-borate buffer (0.2 M boric acid, 0.2 M Tris, 10 mM Na_2 EDTA, pH 7.6, with 0.5% sodium dodecyl sulfate and 0.5% 2-mercaptoethanol) was added, and the cells were lysed at 55 C for 10 min in a heating block. The phenol extractions were omitted after the lysis step, and the samples were spun for 20 min at 10,000 *g* at 4 C to pellet the debris. Thirty microliters of 1 M Na acetate-acetic acid buffer, pH 4.5, was added to 500 μl of the cleared supernatant along with 200 μl of 2-butoxyethanol to selectively precipitate carbohydrate and phenolic impurities. After 1 h on ice, the samples were centrifuged for 10 min at 20,000 *g* at 4 C. The supernatant was carefully transferred to a new tube, and 300 μl of 2-butoxyethanol was added. The nucleic acids were precipitated 1 h on ice. The nucleic acid pellets obtained after centrifuging 10 min at 20,000 *g* were resuspended in Tris-EDTA (TE) buffer and extracted twice with phenol and chloroform and once with chloroform. Nucleic acid was then precipitated overnight at −20 C with sodium acetate and ethanol. Pellets were resuspended in a final volume of 500 μl

TABLE 1. Infection of *Theobroma cacao* with a monomeric DNA clone of cacao swollen shoot virus by microprojectile bombardment

Inoculum[a]	Number of plants with symptoms[b]/total inoculated	
	Experiment I	Experiment II
No treatment	0/5	0/8
pBluescript	ND[c]	0/8
2P-14 unrestricted	ND	0/9
S-2 restricted with *Sma*I	ND	0/7
2P-14 restricted with *Pst*I	30/34	13/20

[a] Cacao seeds from which the testae had been removed were bombarded in pairs with 1 μg of plasmid or recombinant DNA. The two plasmid clones, 2P-14 and S-2, contained one copy of the viral genome inserted into the *Pst*I and *Sma*I polylinker sites, respectively. Plasmid clone 2P-14 was derived from the genomic plasmid clone S-2 by subcloning.

[b] Plants were observed for 18 wk. Symptoms included red veinbanding, mosaic, chlorosis, fern-leaf mosaic on leaves, and swellings on root and stem.

[c] Not done.

of TE. After heat denaturation at 100 C for 10 min, 60 μl was passed through a positively charged nylon membrane. The membrane was UV fixed on both sides.

A randomly primed, digoxigenin-labeled DNA probe was made with *Hae*III-restricted S-2 as template according to the manufacturer's instructions. Prehybridization and hybridization were done in 10 ml of 7% sodium dodecyl sulfate, 0.75 M NaCl, 0.075 M sodium citrate, 2% blocking reagent, 50 mM sodium phosphate, pH 7.0, and 0.1% *N*-lauroylsarcosine at 54 C. Detection was as described by Holtke et al (15).

Immunosorbent electron microscopy (ISEM). Chlorotic leaf tissue was extracted as described by Adomako et al (1). Leaf tissue (10 mg) was ground with 20 mg of Carborundum in 320 μl of extraction buffer (0.05 M phosphate, 5 mM dithiothreitol, 5 mM sodium diethyldithiocarbamate, 0.5% polyethylene glycol 6000, 1% pectinase, pH 6.1) in watch glasses and incubated for 2 h at 37 C prior to clarification. Pyroxylin-filmed grids were coated with a 1/2,000 dilution of antiserum for 30 min at 37 C and rinsed twice for 10 min at room temperature in 60 mM KH_2PO_4-Na_2HPO_4 phosphate buffer, pH 7.2. Particles were trapped for 2 h at 22 C and rinsed as before, and grids were negatively stained with 1% ammonium molybdate, pH 7.0. Observations were done on a CM10 electron microscope (Philips Electronic Instruments, Eindhovon, The Netherlands).

RESULTS AND DISCUSSION

After 9 mo of observation, no symptoms were observed on the 96 mechanically inoculated plants, and dot blot hybridization tests were negative. Although we cannot be sure that the mechani-

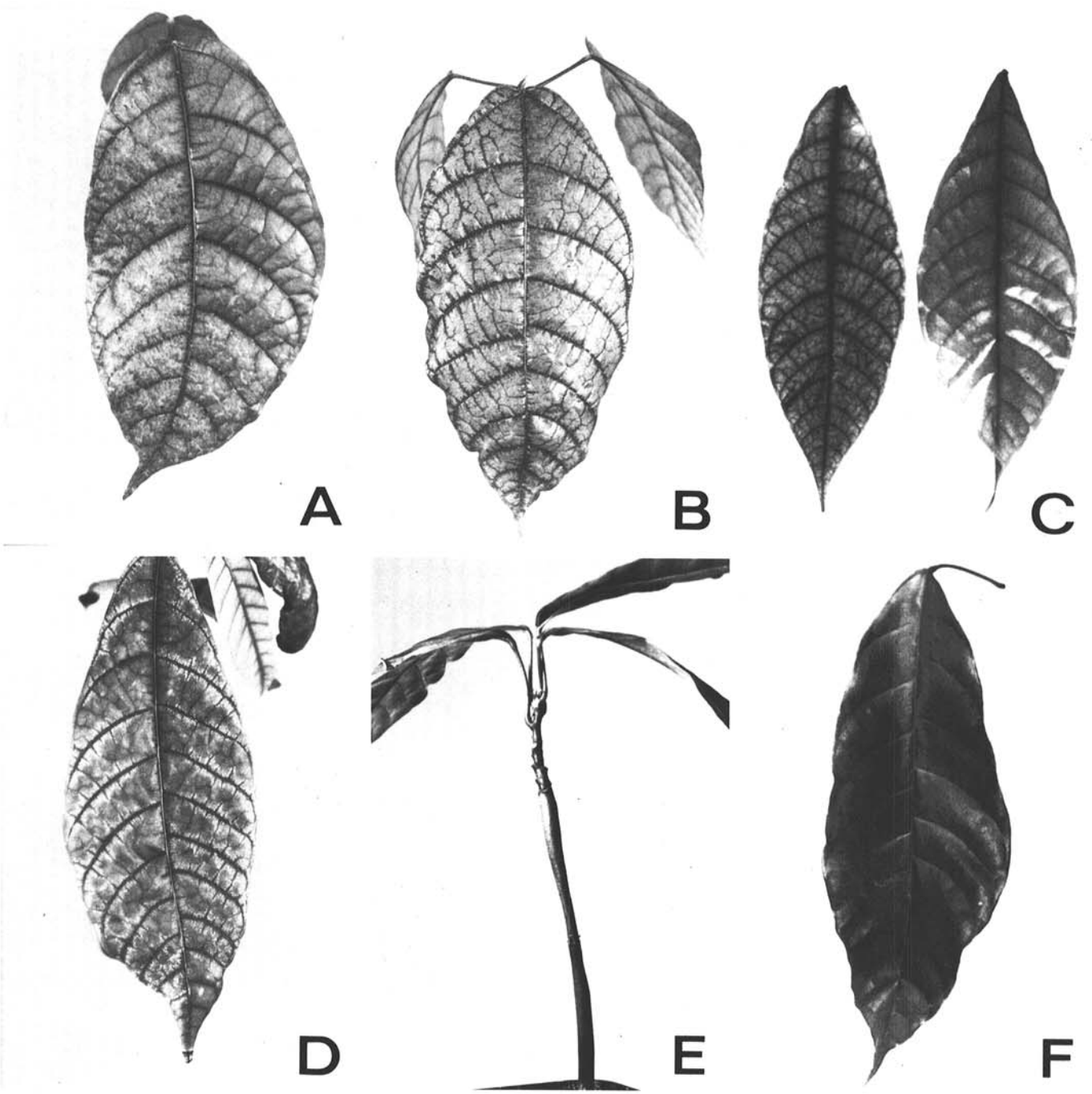

Fig. 1. Cacao swollen shoot symptoms on microprojectile bombardment-inoculated *Theobroma cacao*. Acute symptoms of **A,** fine speckling, **B,** interveinal chlorosis, and **C,** red veins on immature leaf (left) next to an immature healthy leaf (right). Chronic symptoms of **D,** fern-leaf mosaic and **E,** stem swellings. **F,** Mature leaf of a control plant.

cal inoculation conditions were optimal because we did not have at our disposal a positive control of infectious purified virus, the cloned DNA was not infectious under these conditions. In contrast to the caulimovirus and geminivirus groups, where some members can be mechanically transmitted by using naked DNA (native or cloned), no badnavirus DNA has been infectious after mechanical inoculation. CoYMV particles can be mechanically transmitted, but purified DNA at 50 μg/ml can not (21). RTBV particles are not mechanically transmissible and neither is the cloned viral DNA at 250 μg/ml (7). Our result with cloned CSSV DNA is in agreement with this trend.

Biolistic inoculation of cacao seeds with cloned CSSV DNA resulted in 88 and 65% infection in the two separate experiments (Table 1). This is the first successful inoculation by bombardment of DNA from a virus group other than the bipartite geminiviruses with which biolistic inoculations can result in 60–90% infection (9,10). While the efficiency of the bombardment inoculation was satisfactory in both experiments, there was an unexplained difference in the percentage of infection obtained in the two experiments. The same plasmid preparation was used for both experiments, and there are no reports of variation in sensitivity of cacao seed from pods harvested at different seasons. We can only conclude tentatively that the substitution of a rigid sample support with a resilient one, while having no effect on routine experiments on tobacco leaf tissue (C. Godon, *unpublished observation*), decreased the percentage of cocoa seeds infected in the second experiment. Also, the standard target distance of 10 cm used for leaf tissue was less effective than the 7-cm distance used in these experiments (data not shown). It is clear that bombardment parameters must be optimized specifically for inoculation experiments. In addition to the use of whole beans as targets, bombardment of the embryo on half beans or young seedlings could also be tried.

We chose to work with the clone 2P-14 restricted at the *Pst*I cloning site (an excised viral DNA monomer with cohesive termini). This decision was based on work with CaMV, which showed that the viral DNA must be excised from the plasmid to be infectious (16,19) and that infectivity was destroyed if the cohesive termini of the clone were eliminated through mild S1 nuclease digestion (16). For bipartite geminiviruses, Gilbertson et al (10) found it necessary to excise the viral DNA, while Garzón-Tiznado et al (9) were able to infect with circular recombinant plasmids. With CSSV, only seeds inoculated with 2P-14 restricted with *Pst*I were infected; no seeds inoculated with vector alone, undigested 2P-14, or S-2 restricted with *Sma*I (i.e., blunt ends) were infected (Table 1). Therefore, on the basis of this single experiment, it appears that CSSV DNA should be excised and have cohesive termini in order to efficiently establish an infection.

The first symptoms appeared on some plants 5–6 wk after planting when the first leaves hardened. However, symptom expression was variable, and some plants first showed symptoms on second or third leaf flushes (up to the seven-leaf stage). This is similar to the results of experiments in which seeds were inoculated with viruliferous mealybugs (28). Leaves of early flushes had either a fine chlorotic speckling or a blotching (Fig. 1A), speckling over the entire leaf, or interveinal chlorosis (Fig. 1B). Emerging leaves had transient red veins (Fig. 1C). Chronic-phase symptoms of fern-leaf mosaic (Fig. 1D) and stem swellings (Fig. 1E) were first observed after 14 wk. Leaves that emerged later sometimes had edges that curled under and red veins in a fern-leaf pattern that were broader and persistent (not shown). In experiment I at 18 wk, stem swellings were seen in nine of the 30 infected plants, and root swellings (not shown) were found in nine of the 27 infected plants that were uprooted and examined. Thus, symptoms characteristic of cacao swollen shoot disease were reproduced in the bombardment-infected plants. While demonstrating the pathogenicity of the cloned genomic DNA, we have also shown that a single entity can induce the syndrome of swollen shoot disease in its severe form.

ELISA tests were done on all plants in all groups to confirm the infections. Plants that developed symptoms tested positive in the ELISA test. Dot blot hybridization was used to show the presence of viral DNA. Only plants with symptoms were positive. As a means of detecting CSSV, however, hybridization is not more sensitive than ELISA, and because of the extensive sample preparation necessary to eliminate nonspecific signals from healthy plant extracts, the test is too laborious for routine use.

The presence and morphology of the viral particles were evaluated by ISEM. Small bacilliform viral particles were found (Fig. 2) that were 33–346 nm in length (Fig. 3). The modal length and width were 113 and 28 nm, respectively. There is natural variation in particle length, which can result from infection with a single viral clone. However, the extremely long particles reported in some CSSV purifications (1) were not observed. Whether long particles were absent in the bombarded plants or whether they were present but not efficiently trapped on the sensitized grids is not known.

Although purified CSSV is mechanically transmissible to cocoa seeds, it has proven difficult to purify enough inoculum for routine

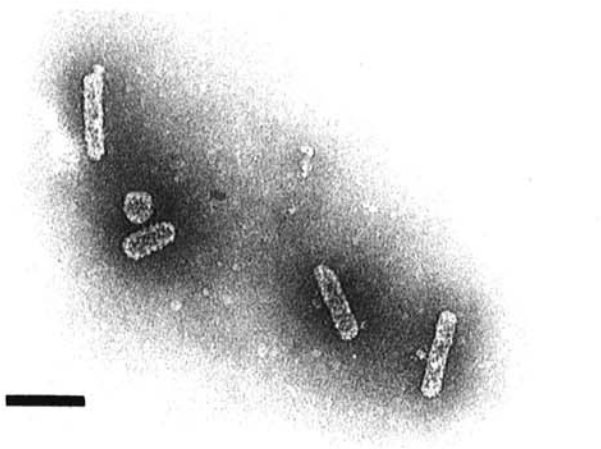

Fig. 2. Electron micrograph of cacao swollen shoot badnavirus particles detected by immunosorbent electron microscopy in crude leaf extracts prepared from bombardment-infected cocoa plants. Particles were trapped on antiserum-coated grids and stained with ammonium molybdate. Bar = 100 nm.

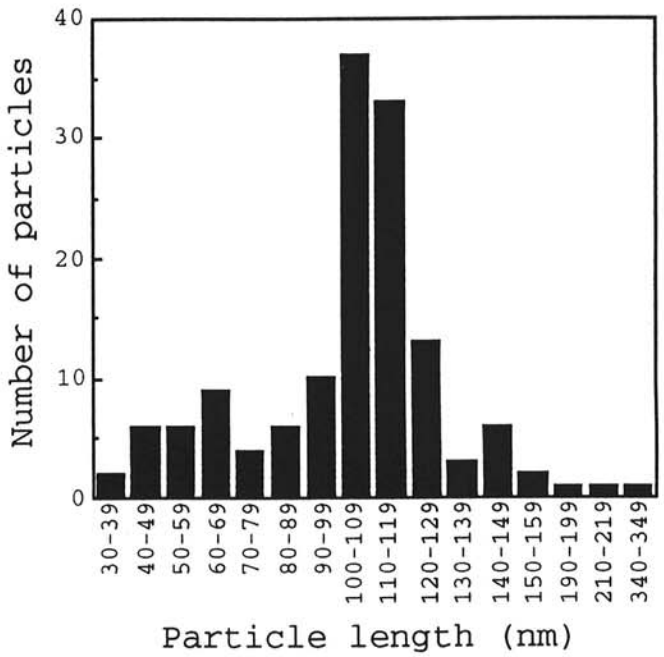

Fig. 3. Length distribution profile of cacao swollen shoot badnavirus particles.

screening for viral resistance (20). Mealybug inoculation of cacao seeds (28) is still the current practice, but it is inconvenient, labor intensive, and difficult to standardize (18). If the bombardment technique were optimized and scaled up to inoculate several seeds with each shot, the technique would be useful for assessing resistance. A large amount of plasmid DNA could be prepared and stored frozen, providing a controlled inoculum. In addition to these aspects, the infectious clone will allow further studies on the molecular aspects of the genome of CSSV.

LITERATURE CITED

1. Adomako, D., Lesemann, D. E., Paul, H. L., and Owusu, G. K. 1983. Improved methods for the purification and detection of cacao swollen shoot virus. Ann. Appl. Biol. 103:109-116.
2. Asomaning, E. J. A., and Lockard, R. G. 1964. Studies on the physiology of cocoa (*Theobroma cacao* L.). 1. Suppression of swollen-shoot virus symptoms by light. Ann. Appl. Biol. 54:193-198.
3. Bouhida, M., Lockhart, B. E. L., and Olszewski, N. E. 1993. An analysis of the complete sequence of a sugarcane bacilliform virus genome infectious to banana and rice. J. Gen. Virol. 74:15-22.
4. Brunt, A. A., and Kenten, R. H. 1960. Mechanical transmission of cocoa swollen shoot virus. Virology 12:328-330.
5. Brunt, A. A., and Kenten, R. H. 1963. The use of protein in the extraction of cocoa swollen shoot virus from cocoa leaves. Virology 19:388-392.
6. Chen, D. F., and Dale, P. J. 1992. A comparison of methods for delivering DNA to wheat: The application of wheat dwarf virus DNA to seeds with exposed apical meristems. Transgenic Res. 1:93-100.
7. Dasgupta, I., Hull, R., Eastop, S., Poggi-Pollini, C., Blakebrough, M., Boulton, M. I., and Davies, J. W. 1991. Rice tungro bacilliform virus DNA independently infects rice after *Agrobacterium*-mediated transfer. J. Gen. Virol. 72: 1215-1221.
8. Evans, D., and Jeske, H. 1993. DNA B facilitates, but is not essential for, the spread of abutilon mosaic virus in agroinoculated *Nicotiana benthamiana*. Virology 194:752-777.
9. Garzón-Tiznado, J. A., Torres-Pacheco, I., Ascencio-Ibañez, J. T., Herrera-Estrella, L., and Rivera-Bustamante, R. F. 1993. Inoculation of peppers with infectious clones of a new geminivirus by a biolistic procedure. Phytopathology 83:514-521.
10. Gilbertson, R. L., Faria, J. C., Hanson, S. F., Morales, F. J., Ahlquist, P., Maxwell, D. P., and Russell, D. R. 1991. Cloning of the complete DNA genomes of four bean-infecting geminiviruses and determining their infectivity by electric discharge particle acceleration. Phytopathology 81:980-985.
11. Godon, C., Caboche, M., and Danielvedele, F. 1993. Transient plant gene expression—A simple and reproducible method based on flowing particle gun. Biochimie 75:591-595.
12. Grimsley, N. 1990. Agroinfection. Physiol. Plant. 79:147-153.
13. Hagen, L. S., Jacquemond, M., Lepingle, A., Lot, H., and Tepfer, M. 1993. Nucleotide sequence and genomic organization of cacao swollen shoot virus. Virology 196:619-628.
14. Hamilton, W. D. O., Bisaro, D. M., Coutts, R. H. A., and Buck, K. W. 1983. Demonstration of the bipartite nature of the genome of a single-stranded DNA plant virus by infection with the cloned DNA components. Nucleic Acids Res. 11:7387-7396.
15. Holtke, H. J., Sagner, G., Kessler, C., and Schmitz, G. 1992. Sensitive chemiluminescent detection of digoxigenin-labeled nucleic acids: A fast and simple protocol and its applications. BioTechniques 12:104-113.
16. Howell, S. H., Walker, L. L., and Dudley, R. K. 1980. Cloned cauliflower mosaic virus DNA infects turnips (*Brassica rapa*). Science 208:1265-1267.
17. Hughes, d'A. J., and Ollennu, L. A. 1993. The virobacterial agglutination test as a rapid means of detecting cocoa swollen shoot virus. Ann. Appl. Biol. 122:299-310.
18. Kenten, R. H., and Legg, J. T. 1970. Methods for assessing the tolerance and resistance of different types of cocoa to cocoa swollen-shoot virus. Ann. Appl. Biol. 65:419-424.
19. Lebeurier, G., Hirth, L., Hohn, T., and Hohn, B. 1980. Infectivities of native and cloned DNA of cauliflower mosaic virus. Gene 12:139-146.
20. Legg, J. T., and Lockwood, G. 1977. Evaluation and use of a screening method to aid selection of cocoa (*Theobroma cacao*) with field resistance to cocoa swollen-shoot virus in Ghana. Ann. Appl. Biol. 86:241-248.
21. Lockhart, B. E. L. 1990. Evidence for a double-stranded circular DNA genome in a second group of plant viruses. Phytopathology 80:127-131.
22. Longworth, J. F., and Thresh, J. M. 1963. The reaction of different cacao types to infection with swollen-shoot virus. Ann. Appl. Biol. 52:117-124.
23. Lot, H., Djiekpor, E., and Jacquemond, M. 1991. Characterization of the genome of cacao swollen shoot virus. J. Gen. Virol. 72:1735-1739.
24. Manning, K. 1991. Isolation of nucleic acids from plants by differential solvent precipitation. Anal. Biochem. 195:45-50.
25. Medberry, S. L., Lockhart, B. E. L., and Olszewski, N. E. 1990. Properties of *Commelina* yellow mottle virus's complete DNA sequence, genomic discontinuities and transcript suggest that it is a pararetrovirus. Nucleic Acids Res. 18:5505-5513.
26. Partiot, M. 1983. Le swollen shoot du cacaoyer (*Theobroma cacao:* Sterculiacees): Etude de la nature et de l'evolution d'un couple hote-parasite dans un ecosysteme tropical. Ph.D. diss. Université Paris-Sud, Centre Orsay, France.
27. Posnette, A. F. 1947. Virus diseases of cacao in West Africa. I. Cacao viruses 1A, 1B, 1C and 1D. Ann. Appl. Biol. 34:388-402.
28. Posnette, A. F., and Strickland, A. H. 1948. Virus diseases of cacao in West Africa. III. Technique of insect transmission. Ann. Appl. Biol. 35:53-63.
29. Posnette, A. F., and Todd, J. M. 1955. Virus diseases of cacao in West Africa. IX. Strain variation and interference in virus 1A. Ann. Appl. Biol. 46:433-453.
30. Roivainen, O. 1976. Transmission of cocoa virus by mealybugs. J. Sci. Agric. Soc. Finl. 48:203-304.
31. Sagemann, W., Lesemann, D. E., Paul, H. L., Adomako, D., and Owusu, G. K. 1985. Detection and comparison of some Ghanian isolates of cacao swollen shoot virus (CSSV) by enzyme-linked immunosorbent assay (ELISA) and immunoelectron microscopy (IEM) using an antiserum to CSSV strain 1A. Phytopathol. Z. 114:79-89.
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. Sanford, J. C., Klein, T. M., Wolf, E. D., and Alen, N. 1987. Delivery of substances into cells and tissues using a particle bombardment process. Part. Sci. Technol. 5:27-37.
34. Stanley, J. 1983. Infectivity of the cloned geminivirus genome requires sequences from both DNAs. Nature 305:643.