Partial Characterization and Molecular Cloning of a Closterovirus from Sweet Potato Infected with the Sweet Potato Virus Disease Complex from Nigeria

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Flexuous, filamentous, viruslike particles with a modal length of 950 nm were consistently observed in leaf dip preparations of *Ipomoea setosa* infected with the whitefly-transmitted component of the sweet potato virus disease (SPVD) from Nigeria. Ultrastructural examinations of infected tissue from *I. setosa* revealed membrane-enclosed vesicles containing fibrillar material in the phloem parenchyma, which are indicative of closterovirus infections. These structures were also present in thin sections prepared from SPVD-infected *I. setosa* tissue. Analysis of dsRNA extracts revealed the presence of a large dsRNA (molecular weight about 6.1 $\times 10^6$) and several smaller dsRNA species in all sweet potato and *I. setosa* plants carrying the whitefly-transmitted component (WTA). Synthesis and cloning of cDNA using dsRNA isolated from WTA-infected sweet potato as template resulted in clones that specifically reacted with nucleic acid prepared from purified closterovirus as well as with RNA isolated from plants infected with the WTA. In Northern blot analysis a hybridization signal to an ssRNA with an estimated size of about 9 kb was obtained, which in concordance with the size of the largest dsRNA species found and the length of the closteroviruslike particles, was referred to as virion RNA. The data suggest that a closterovirus and sweet potato feathery mottle virus are the causal agents of sweet potato virus disease in Nigeria. Although definite proof of the whitefly transmissibility of the WTA is still lacking, evidence for the existence of other agents involved in the SPVD complex could not be substantiated.

Sweet potato virus disease (SPVD) was described by Schaefers and Terry (26) in Nigeria and is known to be caused by the interaction between sweet potato feathery mottle potyvirus (SPFMV) and a whitefly-transmitted agent (WTA) of possible viral origin. Single infection with the WTA remains symptomless in *Ipomoea batatas* (L.) Lam.; therefore, on the basis of symptoms expressed in *I. setosa* Ker. indicator plants, the whitefly-transmitted component of SPVD was referred to as sweet potato chlorotic stunt (SPCS).

Although much effort has been put into the characterization of that pathogen (13,15,24,26), especially of its whitefly transmissibility and host range, the etiology of SPVD still remains unknown. Other whitefly-transmitted agents contributing to a disease syndrome in sweet potato resembling that of SPVD have been reported from various countries (5,14,17,23,28). But, if it could be shown that these agents not only share the same vector but are also isolates or strains of the same pathogen, then the WTA would attain significance beyond that of a pathogen with limited distribution.

The lack of information on the nature of the WTA renders disease indexing cumbersome. The accepted method of WTA diagnosis is by index grafting on SPFMV-infected *I. batatas* (20), thus establishing the SPVD disease complex. Although accurate, this method is laborious and inefficient. Therefore, identification and characterization of the WTA would contribute to the development of an indexing procedure which, by replacing existing methods, would represent a substantial improvement in programs that certify plants as free of virus and in quarantine testing, thus removing a significant impediment to the international exchange of sweet potato germ plasm.

This research project was initiated to identify the whitefly-transmitted component of the SPVD complex from Nigeria and to develop a rapid and reliable indexing method for this component. In this report we describe the isolation and partial characterization of a closterovirus found together with SPFMV in plants infected with SPVD.

MATERIALS AND METHODS

Virus isolates and plant materials. All sweet potato plant materials used for the experiments were obtained from germ plasm accessions at IITA, Nigeria. At IITA, healthy sweet potato seedlings grown from true seed and clonally propagated in isolation were used as virus recipients. WTA-infected sweet potato plants were obtained by whitefly transmission from SPVD-diseased sweet potato to a healthy *I. setosa* seeding and from there, by graft transmission, to sweet potato TIb 10 (tropical *I. batatas* 10). The SPFMV-infected TIb 8 clone was established from a field-collected cutting from a nonsymptomatic sweet potato plant that tested positive for SPFMV by index grafting to *I. setosa*. In Vancouver, the sweet potato clones were propagated as rooted stem cuttings and kept under greenhouse conditions at 25 C or above. Presence of SPFMV and of the WTA in their respective TIb clones was confirmed by index grafting. Scions of TIb 8 were grafted onto *I. setosa* plants and typical symptoms of SPFMV were observed 2–3 wk after inoculation. SPFMV infection of the indicator plants was confirmed by ELISA (antiserum courtesy of J. W. Moyer) and by electron microscopy. Scions of TIb 10 were cleft-grafted onto SPFMV-infected TIb 8 and *I. setosa*, and symptoms of SPVD developed after 2 wk in *I. setosa* and 3–5 wk after graft inoculation in *I. batatas*. Healthy TIb plants were repeatedly tested by index grafting and kept as noninfected control plants.

Electron microscopy. Preparations of purified virus and homogenates of excised leaf midrib tissue from various plants infected with the WTA either in single infection or within the SPVD complex were negatively stained with 2% phosphotungstate, pH 6.8, and then examined by electron microscopy. For ultrathin sections, leaf midvein tissue was excised and fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After postfixation in buffered 2% osmium tetroxide, the tissue was de-

hydrated in a graded ethanol series and then embedded in Epon using propylene oxide. Ultrathin sections were contrasted using 4% aqueous uranyl acetate and lead citrate (6).

Virus purification. Source plants were *I. setosa* that had been graft-inoculated with WTA-infected TIb 10 scions about 6–8 wk before the extraction date. The plants were cut above the graft insertion, pulverized in liquid nitrogen, and transferred to a stirring solution (1:4, w/v) of homogenization buffer (0.5 M Tris-HCl, pH 8.2, 2% water-insoluble polyvinyl pyrollidone, 0.2% 2-mercaptoethanol, 4% Triton X-100). After it was stirred for 30 min at 4 C, the extract was clarified by low-speed centrifugation, and the supernatant fluid was adjusted with polyethylene glycol (PEG, MW 6,000) to 6% (w/v) and with NaCl to 0.2 M, and agitated for 1.5 h at 4 C. The precipitate was collected by centrifugation (8,000 *g*, 20 min), and the pellets were dissolved by shaking overnight in TM buffer (0.1 M Tris, pH 8.2, 0.01 M $MgCl_2$) containing 10% sucrose (TMS). After clarification (3,000 *g*, 5 min), the virus suspension was concentrated by high-speed centrifugation (96,000 *g*, 2 h) through a 20% sucrose cushion in TM buffer. Pellets were dissolved in TMS buffer and subjected to another cycle of differential centrifugation. Partially purified virus was layered on a preformed 5–30% (w/w) $CsSO_4$ gradient buffered in TMS and centrifuged at 160,000 *g* for 3 h at 10 C in a Beckman SW41 rotor. Fractions containing viruslike, filamentous particles, determined by electron microscopy, were pooled, diluted in TMS, and subjected to another $CsSO_4$ gradient centrifugation. After a final high-speed centrifugation, the virus pellet was resuspended in TMS and examined by electron microscopy and by electrophoresis through 12% polyacrylamide-sodium dodecyl sulfate (SDS) gels (25).

Nucleic acid preparations. RNA from purified closterovirus was isolated by diluting pooled fractions of the $CsSO_4$ gradient 1:2 (v/v) in 20 mM Tris, pH 9.0, and adding EDTA to 10 mM to destabilize the virus particles. The virus preparation was placed on ice for 5 min, then adjusted to 200 mM sodium acetate, pH 5.0, 0.5% SDS, 200 μg/ml proteinase K and incubated for 3 h at 37 C. After phenol/chloroform treatment, RNA was precipitated with 2.5 vol ethanol in the presence of 0.3 M sodium acetate, pH 5.3.

To obtain total leaf RNA from infected and healthy control plants, the acid guanidinium thiocyanate procedure (4) was used with some minor modifications.

DsRNA extractions from whole plant tissue were done essentially as described by Morris and Dodds (19). After phenol/chloroform treatment, tissue extracts were subjected to two cycles of Whatman CF11 column chromatography followed by ethanol precipitation. Subsequently, nucleic acid samples were treated with RNase T1 (0.1 units/μl) and DNase I (0.1 units/μl) followed by proteinase K digestion (200 μg/ml), phenol/chloroform treatment, and ethanol precipitation. Analysis of dsRNA was done in 6% polyacrylamide gels buffered with TAE (40 mM Tris, pH 7.8, 20 mM sodium acetate, and 1 mM EDTA). DsRNA extracted from about 20 g of tissue (fresh weight) was loaded on each lane and electrophoresis was performed at 100 V for 4 h. After electrophoresis, dsRNA gels were stained with silver nitrate using a modification of the method described by Schumacher et al (27).

Total genomic DNA from *I. batatas* and *I. setosa* was extracted following the hexadecyltrimethylammonium bromide (CTAB) protocol described by Doyle and Doyle (8).

Northern blot analysis. For Northern blot analysis, ssRNA and dsRNA were denatured in formamide/formaldehyde and electrophoresed in 20 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS)-buffered agarose gels containing formaldehyde (25). RNA was partially hydrolyzed in dilute alkali and then transferred to a nylon membrane (Zeta-Probe GT, Bio-Rad, Richmond, CA) by vacuum blotting in 10× SSC for 45 min at 30 mm of Hg. DsRNA was fractionated under nondenaturing conditions in 1% TAE-buffered agarose gels and transferred to a nylon membrane under alkaline conditions (50 mM NaOH). Prior to nucleic acid fixation, the membrane was soaked for 10 min in 400 mM NaOH. Membrane preparation, for dot blot hybridization of DNA and RNA samples as well as for Northern blots, nucleic acid fixation, prehybridization, hybridization, and washing steps was as described by the manufacturer (Bio-Rad). To determine the size of RNA, the position of the observed Northern blot signal was compared with the migration distances of the 0.24–9.5 kb single-stranded RNA size standards (BRL), separated in the denaturing gel.

cDNA synthesis and molecular cloning. Approximately 500 ng of dsRNA derived from WTA-infected TIb 10 was used as template for cDNA synthesis. Prior to first-strand synthesis, dsRNA and primers, both oligo dT_{12-18} (350 ng) and pdN_6 (200 ng, Pharmacia, Uppsala, Sweden), were denatured in 20 mM methylmercuric hydroxide in a volume of 10 μl for 10 min at room temperature. First- and second-strand synthesis was carried out in one tube using Moloney murine leukemia virus RNase H^- reverse transcriptase (M-MLV H^- RT, GIBCO BRL) for first-strand synthesis, followed by DNA polymerase I-catalyzed second-strand replacement synthesis in the presence of RNase H and *Escherichia coli* DNA ligase (11). After phenol/chloroform treatment, synthetic dsDNA was size-fractionated by chromatography on Sepharose CL-4B (Pharmacia), ethanol precipitated, washed with 70% ethanol, and dried. Homopolymeric tailing of the dsDNA was done using dCTP as substrate, and the resulting tailed product was annealed to oligo(dG)-tailed *Pst*I-digested pUC9 vector (Pharmacia). MAX efficiency, DH5α competent *E. coli* cells (BRL) were transformed following the manufacturer's protocol and plated onto LB agar containing ampicillin and the chromogenic substrate, X-Gal. White bacterial colonies were picked and grown in small-scale cultures. Plasmid DNA was isolated by a standard alkaline lysis minipreparation method (25)

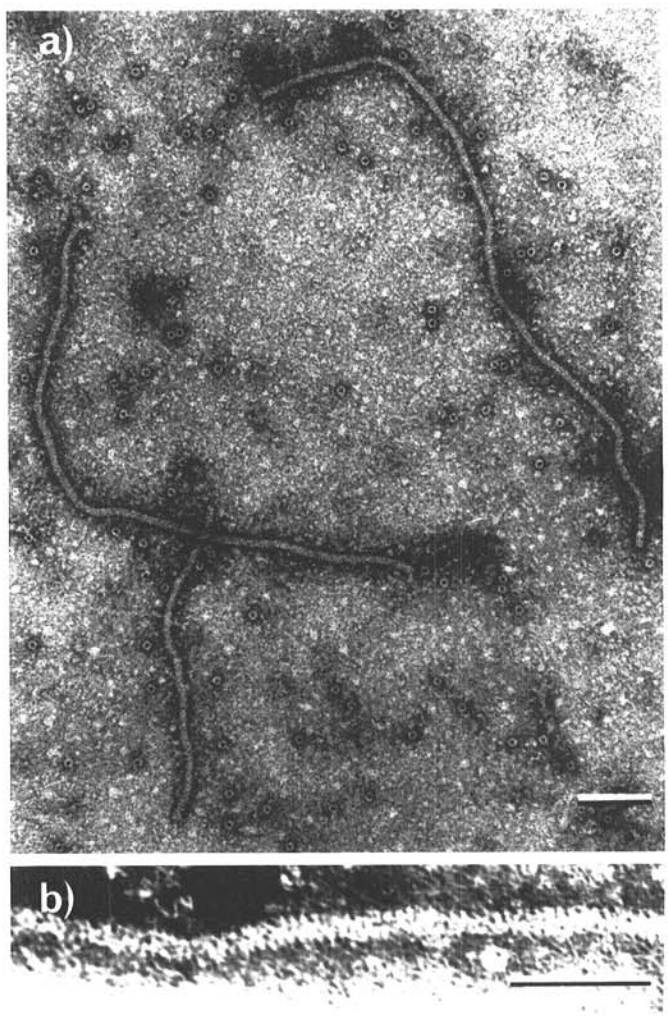

Fig. 1. Electron micrograph of closteroviruslike particles in dip preparations of leaf midvein tissue from WTA-infected *Ipomoea setosa*. Bars = 100 nm in **A** and 50 nm in **B**.

and analyzed for the presence of recombinant cDNA by *Pst*I restriction endonuclease digestion and agarose electrophoresis.

Library screening. A primary screening procedure eliminated clones containing plant nucleic acid sequences. Recombinant plasmid DNA (500 ng) was heat denatured in alkali and spotted onto Zeta-Probe GT membrane (Bio-Rad). *Hin*dIII-cleaved, radiolabeled total plant genomic DNA (0.5×10^6 cpm/ml hybridization solution) was used for the hybridization analysis, and those clones that gave rise to hybridization signals were discarded. Clones which tested negative by this prescreening procedure were further examined by hybridization of radiolabeled insert DNA in Northern blot analysis against RNA of purified closterovirus and total RNA extracted from healthy and WTA-infected TIb 10 and *I. setosa*.

Probe labeling and hybridization assays. To synthesize cDNA probes for the assays, inserts were excised from plasmid with *Pst*I, separated by agarose gel electrophoresis, and isolated from agarose gels by sodium iodate/glass milk purification (31). Radiolabeling was done according to Feinberg and Vogelstein (10) using random hexa-deoxynucleotide primers (pdN_6, 250 μg/ml, Pharmacia). The labeled probe (10^8–10^9 cpm/μg) was separated by Sephadex G-50 chromatography, boiled, and added to the hybridization solution to give between 0.5 and 1×10^6 cpm/ml. Radiolabeling of *Hin*dIII-cleaved total genomic DNA was done accordingly.

RESULTS

Symptoms. Sweet potato clones TIb 8 and TIb 10 were symptomless when infected with SPFMV and WTA, respectively. Symptoms induced by SPFMV on the *I. setosa* indicator plant consisted of chlorotic mottling, veinclearing, and leaf deformation followed by a general recovery phase, symptoms typical of SPFMV infection (21). *I. setosa* infected with the WTA by graft inoculation showed reduced growth, characterized by shorter internodes (stunting) and smaller, brittle leaves. An inward rolling of the leaves could also be observed. This syndrome developed 3–5 wk after inoculation, when respective control plants already had recovered from the grafting treatment. Typical symptoms of SPCS (26) consisting of yellowing and stunting were observed only under the most favorable conditions, when greenhouse temperatures were kept between 25 and 35 C, under reduced light (in the summer months).

The most striking symptoms of SPVD in TIb 10 and TIb 8 consisted of leaf puckering, vein chlorosis, and leaf distortion. Plants appeared severely dwarfed, and the formation of long shoots was restrained. On the smaller, heavily distorted, and malformed leaves of SPVD-infected *I. setosa*, a wide spectrum of chlorotic leaf symptoms appeared in the initial stage followed by severe epinasty in the chronic phase where only midvein tissue was produced.

Particle morphology and cytopathology. Flexuous, rod-shaped viruslike particles were consistently found in leaf midrib tissue preparations of WTA-infected *I. setosa* (Fig. 1A). They occurred in very low numbers (≤1/grid opening) and varied considerably in length. Measurements in separate experiments of particles found in leaf dips and of purified filaments showed a length distribution in which most of the particles measured between 900 and 1,000 nm (Fig. 2) with a modal length of about 950 nm. The morphology of these filamentous particles showed the typical cross-banding (Fig. 1B) that is characteristic for closteroviruses (1,30). Viruslike particles similar to those found in *I. setosa* were also observed when purified preparations from WTA-infected TIb 10 were examined in electron microscopy.

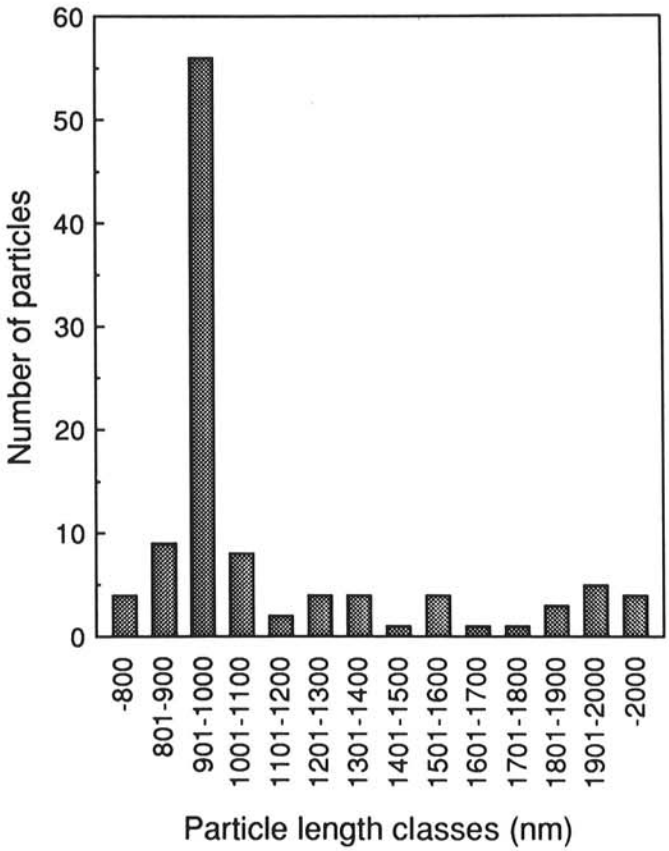

Fig. 2. Measurements of closteroviruslike particles purified from WTA-infected *Ipomoea setosa*.

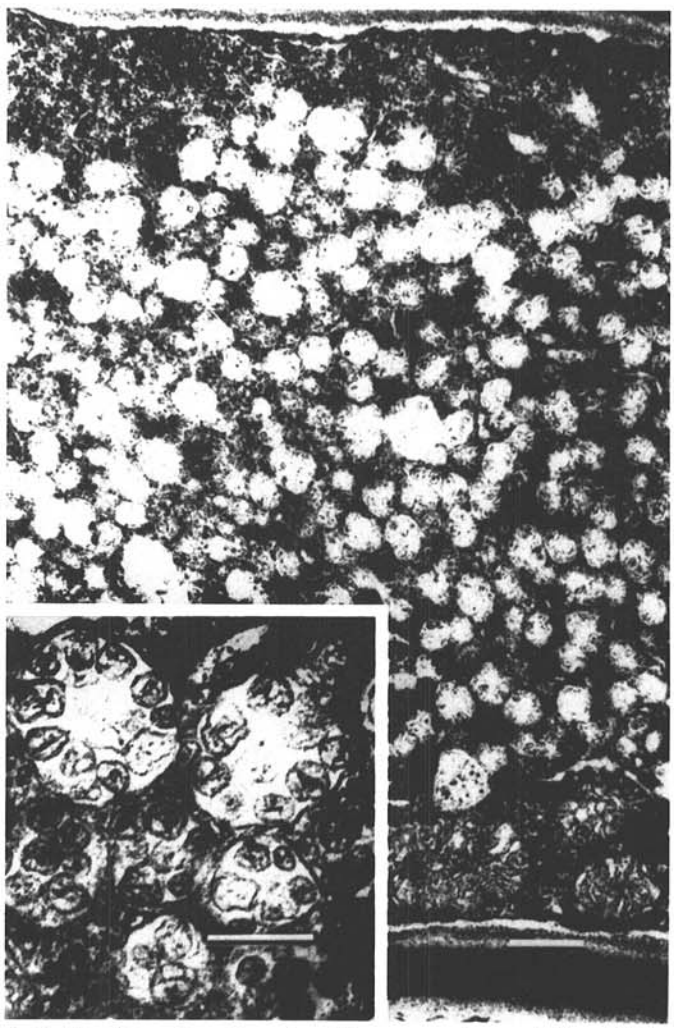

Fig. 3. Ultrathin section through a phloem parenchyma cell from WTA-infected *Ipomoea setosa* showing massive accumulation of vesicle clusters enclosed in a membrane (inset). Bars = 500 nm and 200 nm in inset.

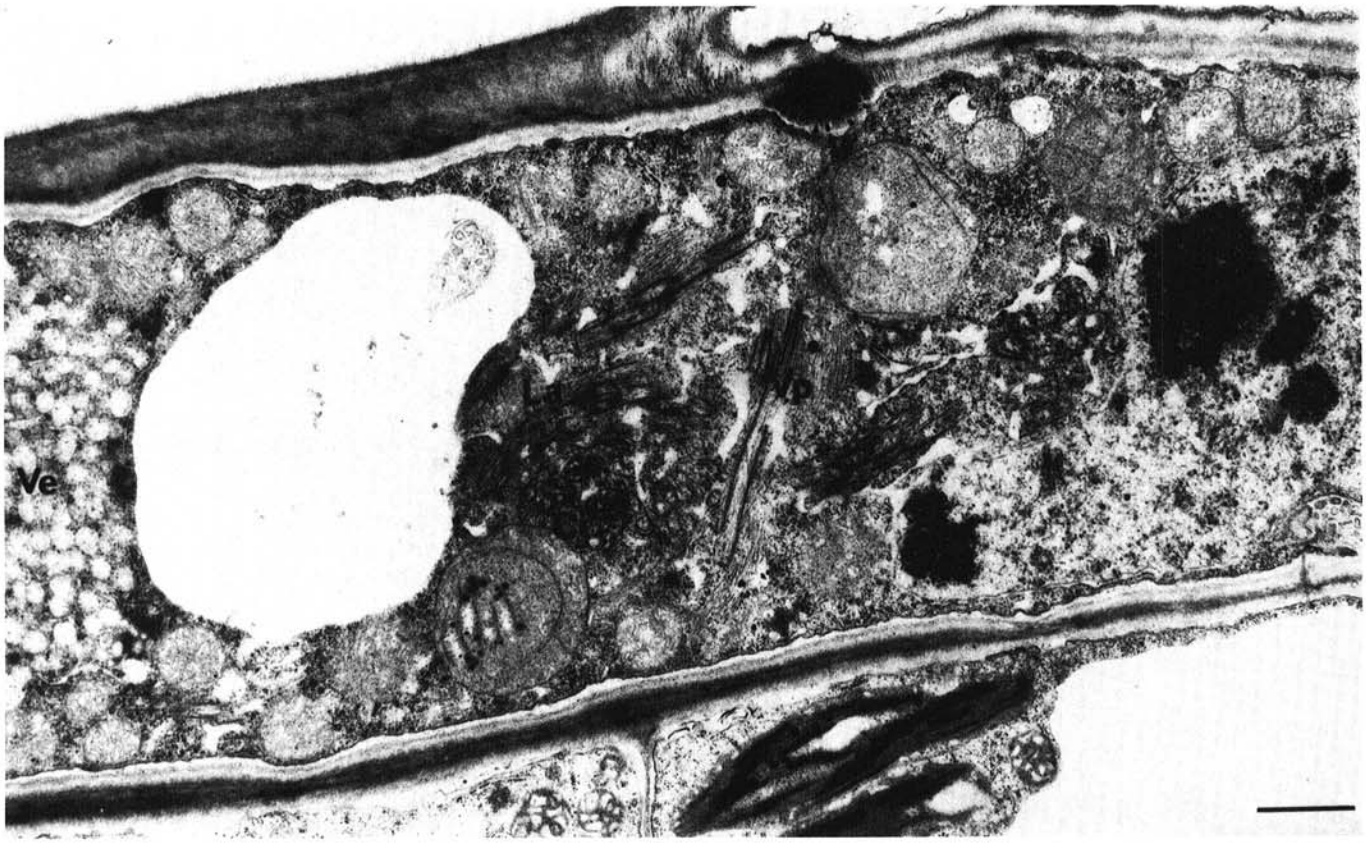

Fig. 4. Ultrathin section through phloem parenchyma cell of *Ipomoea setosa* infected with the sweet potato virus disease complex (SPVD). Membrane-enclosed vesicles (Ve) characteristic of closterovirus infection and cylindrical inclusions (Ci), scrolls and laminated aggregates (La) characteristic of potyvirus infection, and viruslike particles (Vp) are found within the same cell. Bar = 1 μm.

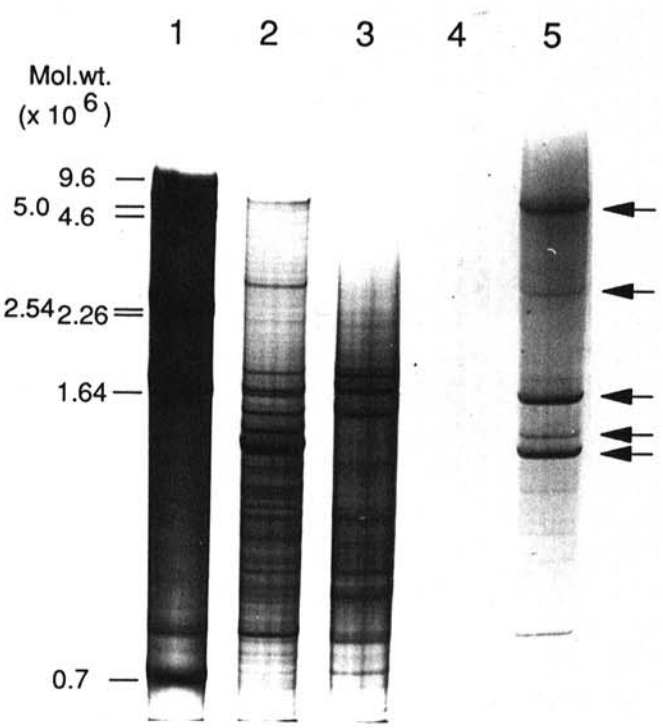

Fig. 5. Polyacrylamide gel electrophoresis of dsRNA extracted from healthy or WTA-infected plants. Lane 1, molecular weight standards consisting of a mixture of dsRNAs from *Phaseolus vulgaris* 'Black Turtle Soup', and from *Nicotiana tabacum* 'Samsun' infected with potato virus X (PVX) and cucumber mosaic virus (CMV), respectively; lane 2, dsRNA from WTA-infected *Ipomoea batatas* TIb 10; lane 3, dsRNA from healthy TIb 10; lane 4, dsRNA from healthy *I. setosa*; lane 5, dsRNA from WTA-infected *I. setosa*. Arrows on the right indicate disease-specific dsRNA bands.

Ultrastructural examination of thin sections prepared from WTA-infected *I. setosa* showed cytoplasmic alterations similar to those induced by members of the closterovirus group (2,16). Numerous small vesicles were found in phloem parenchyma cells (companion cells) occurring in clusters within membranous compartments (Fig. 3) and containing fine, densely stained, fibrillar material. Electron-dense granular material was often associated with the vesicles. In SPVD-infected *I. setosa* tissue, the described membrane-enclosed vesicles together with the typical cylindrical inclusions specific for potyvirus infection (9) could be observed within the same phloem companion cell (Fig. 4). None of the cytoplasmic alterations were observed in ultrathin sections of TIb 10 tissue infected with the WTA.

Virus purification. The procedure described by Zee et al (34) was modified by including a PEG precipitation step. Virus yields were consistently very low, rendering accurate quantification impossible. Besides particle loss due to aggregation, the copurification of endogenous tubular structures contributed to the difficulties in obtaining pure preparations of acceptable virus yield. The production of an antiserum was therefore not pursued. SDS-polyacrylamide gel electrophoretic analysis showed numerous protein bands present in virus preparations. In addition to the banding pattern characteristic of preparations from noninfected control material, a peptide band with an approximate molecular weight of 25–27 kDa was visible (not shown). It was tentatively inferred as the coat protein, although the unequivocal proof can only be given in Western blots using WTA-specific antibodies.

Analysis of dsRNA. Electrophoretic analysis of dsRNA isolated from WTA-graft inoculated *I. setosa* and WTA-infected TIb 10 revealed the presence of various dsRNAs not present in healthy control plants (Fig. 5). Three intensely stained and two minor dsRNA bands were identified as being specific for a WTA infection. Their molecular weights were calculated using the graphical method of Bozarth and Harley (3) by comparing the electrophoretic mobility with similar extracted dsRNAs of bean (*Phaseolus vulgaris* 'Black Turtle Soup') (9.6×10^6; 32), potato virus

X (PVX, 5.0, 4.6 × 10^6; 29), and cucumber mosaic (CMV, 2.54, 2.26, 1.64, 0.7 × 10^6; 33). The estimated molecular weights of the infection-specific dsRNAs were 6.1 × 10^6, 1.65 × 10^6, and 1.3 × 10^6 for the intensely stained bands and 2.92 × 10^6, and 1.4 × 10^6 for the two minor bands. DsRNA profiles obtained from SPVD-infected TIb 10 and TIb 8 were similar to those obtained from WTA-infected tissue; however, an additional dsRNA of higher molecular weight (approximately 7.2 × 10^6) was also found, which corresponds to the length of the genomic RNA of SPFMV.

Synthesis and cloning of cDNA. The dsRNA preparations still contained plant nucleic acids (Fig. 5) that could not be removed by extensive washing during CF-II chromatography and subsequent treatment with either RNase T1 or RNase A. As expected, most of the cDNA clones obtained hybridized with plant genomic DNA. The WTA-specific cDNA clones pCL46 (1.4 kbp), pCL43 (0.8 kbp), and pCL26 (0.8 kbp), which do not overlap, were used as probes in Northern blot analysis of a dsRNA preparation of WTA-infected *I. setosa* that was separated under nondenaturing conditions. The cDNA clone pCL46 hybridized only with the largest dsRNA, whereas pCL43 and pCL26 hybridized also with smaller WTA-specific dsRNA species (Fig. 6). The smallest dsRNA band was, however, only detected in the hybridization with pCL26.

Northern blot analysis. For these experiments the cDNA clone pCL46 was used as probe. DsRNA extracts of plants infected with the WTA or SPFMV in either single or double infection and separated under nondenaturing conditions revealed a hybridization signal with the large dsRNA (about 9 kbp) present in samples containing the WTA (Fig. 7). A nonspecific reaction with dsRNA preparations of healthy or SPFMV-infected plants was never observed. As shown in Figure 7, the largest WTA-specific dsRNA is also clearly distinct in size from the dsRNA that represents the double-stranded replicative form of SPFMV (about 11 kbp).

In Northern blot experiments with nucleic acid from purified closterovirus separated under denaturing conditions, pCL46 hybridized with one major RNA species of about 9 kb (Fig. 8).

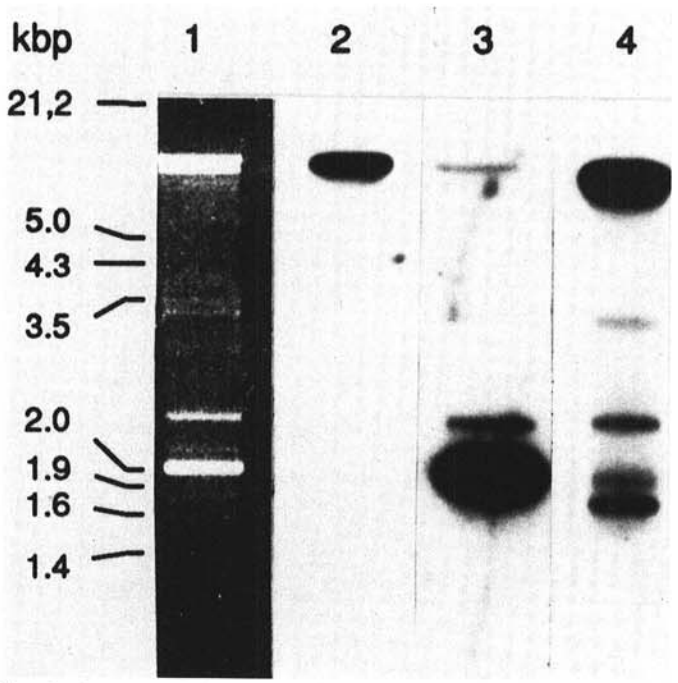

Fig. 6. Agarose gel electrophoresis of dsRNA extracted from WTA-infected *Ipomoea setosa* and Northern blot hybridization with the cDNA clones pCL46, pCL43, and pCL26. DsRNA was fractionated under nondenaturing conditions (lane 1), Northern-transferred and subsequently hybridized with cDNA clones pCL46 (lane 2), pCL43 (lane 3), and pCL26 (lane 4). The positions and sizes (kbp) of lambda DNA *Eco*RI/*Hin*dIII fragments are indicated on the left.

The single-stranded nature of this RNA was determined by its sensitivity to RNase A under low and high salt conditions. Analysis of total leaf RNA fractionated under denaturing conditions and probed with the cDNA clone revealed a hybridization signal with an RNA of similar size (Fig. 8) in nucleic acid extracts from WTA-infected plants. A formamide/formaldehyde-denatured dsRNA sample included in the experiment gave rise to a hybridization signal at exactly the same position.

Dot blot experiments using crude sap and pCL46 DNA as probe confirmed the hybridization obtained in Northern blot analysis (Fig. 9). A nonspecific hybridization with sap from either healthy or SPFMV-infected plants was not detected; however, the signals with sap dots of WTA-infected TIb 10 were only slightly above the background.

DISCUSSION

We have demonstrated that a second filamentous, viruslike particle about 950 nm long is associated with SPFMV in the

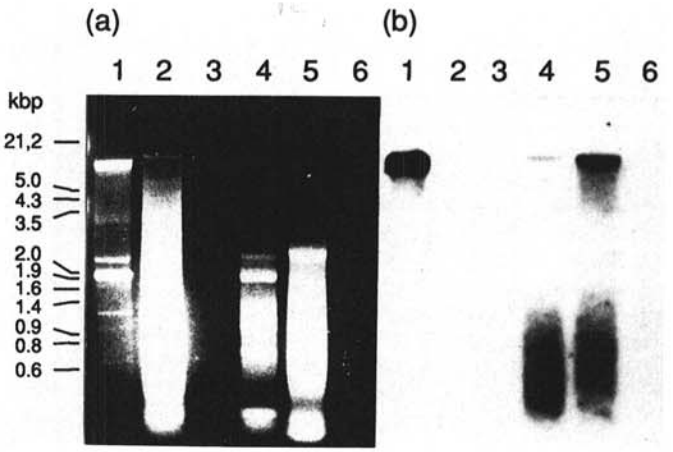

Fig. 7. Agarose gel electrophoresis (A) and Northern blot hybridization (B) of dsRNA extracts from WTA-infected (lane 1), SPFMV-infected (lane 2) and healthy (lane 3) *Ipomoea setosa* and of dsRNA extracts from WTA-infected (lane 4), SPVD-infected (lane 5), and healthy (lane 6) *I. batatas* TIb 10. DsRNA extracts were separated under nondenaturing conditions prior to Northern transfer and hybridization using cDNA clone pCL46 as probe. Numbers in (B) correspond to sample numbers in (A). The positions and sizes (kbp) of lambda DNA *Eco*RI/ *Hin*dIII fragments are indicated on the left. Note, photograph of EtBr stained agarose gel (A) is overexposed to emphasize faint dsRNA band corresponding to replicative form of SPFMV, lane 2.

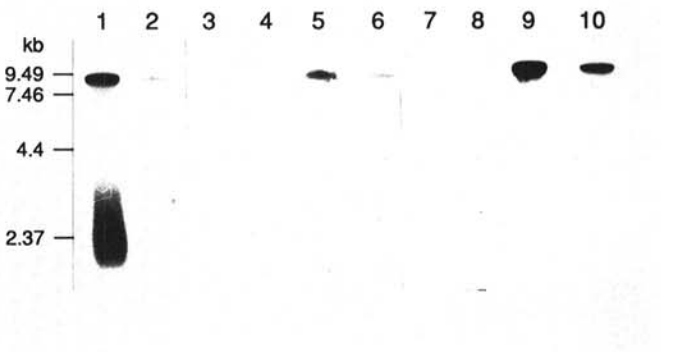

Fig. 8. Northern blot analysis of total RNA extracted from healthy or infected *Ipomoea* spp. after electrophoretic separation under denaturing condititions. DNA clone pCL46 was used as probe. Total leaf RNA from *I. setosa* infected with SPVD (lane 1), WTA (lane 2), SPFMV (lane 3), and from healthy plants (lane 4); total leaf RNA extracted from *I. batatas* TIb 10 infected with SPVD (lane 5), WTA (lane 6) from healthy plants (lane 7) and from *I. batatas* TIb 8 infected with SPFMV (lane 8); ssRNA from purified closterovirus (lane 9), and dsRNA extracted from WTA-infected *I. setosa* (lane 10). Numbers on the left are molecular sizes of the ssRNA standards (BRL).

SPVD complex. These distinctly flexuous, viruslike particles, which also exhibit prominent cross-banding, strongly suggest that they are those of a closterovirus. This conclusion is further strengthened by the presence in WTA- and SPVD-infected *I. setosa* of membrane-bound vesicles in phloem tissue, typical cytopathic alterations found in plants infected with members of the beet yellows virus subgroup of the closteroviruses (18,22). This type of cytopathological effect is specific for this virus group and is, therefore, of diagnostic value (2,16). In SPVD-infected tissue, membrane-enclosed vesicles were observed together with the typical cylindrical inclusions characteristic of potyvirus infections within the same cell.

DsRNA analysis of WTA-infected plants showed the presence of multiple disease-specific bands of various sizes and intensities. For closteroviruses the occurrence of a range of dsRNA species in nucleic acid extracts of infected plants has been described (7,33). In addition to a large dsRNA band (replicative form) of about twice the molecular mass of the ssRNA corresponding to the viral genome, several smaller distinctive virus-specific dsRNA bands of various intensities could be detected. The dsRNA profile that we found to be specific for the incidence of the WTA in infected plants is in good agreement with the reported closterovirus disease-specific dsRNA patterns. We could never observe a host-specific modification of the banding pattern; however, as the somewhat overexposed photograph of the agarose gel in Figure 7 indicates, differences in the relative amounts of the various bands within the dsRNA pattern were always noticeable. These variations are probably explained by the stage of infection and the presence of SPFMV in SPVD-infected plants. The differential hybridization of the cDNA clones pCL46, pCL43, and pCL26 with the dsRNA confirms that the large dsRNA, which we believe is the replicative form of the closterovirus, and the smaller dsRNAs present in WTA-infected plants are specific for this agent. The occurrence of dsRNAs smaller than the genomic length dsRNA might indicate that subgenomic transcripts serve as messenger RNAs in viral replication (12). Subgenomic RNAs represent sequences towards the 3′ end of virion genomic RNA with which they are coterminal. The differential hybridization of the cDNA clones to the smaller dsRNAs is explained by the position of the nucleotide sequences in the RNA to which the cDNA clones are complementary. Lack of hybridization of cDNA clone pCL46 with any of the smaller dsRNAs places the position of its complementary RNA sequence towards the 5′ end of the genome, whereas hybridization of pCL26 to the whole set of dsRNAs indicates that its complementary RNA sequences are near the 3′ end of the viral genome and downstream of sequences complementary to cDNA pCL43 since this clone failed to detect the smallest dsRNA band.

Northern blot experiments with denatured, size-fractionated nucleic acid of purified closterovirus, using cDNA clone pCL46 as probe, resulted in hybridization with one major RNA species with an estimated size of 9 kb. The hybridization signal obtained with denatured dsRNA fractionated under the same conditions substantiates the determination of the genome size of this closterovirus. It furthermore proves that the dsRNA with the molecular weight of about 6.1×10^6 (size of about 9 kbp) found in WTA-infected plants to which pCL46 hybridizes exclusively, is the replicative form of this virus. Considering the similar ratio of RNA mass to virion modal length among closteroviruses (1), the limits of variation due to the stain used and number of counted particles, the molecular weight estimation of genomic RNA and particle measurements in electron microscopy are in close agreement.

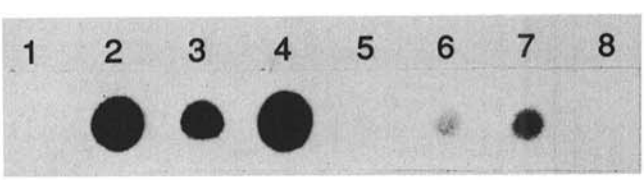

Fig. 9. Dot blot hybridization of cDNA clone pCL46 to sap extracts from healthy *Ipomoea setosa* (1), to RNA prepared from purified closterovirus (2), to sap extracts from WTA-infected (3) SPVD-infected (4), and SPFMV-infected (5) *I. setosa* and to sap extracts from WTA-infected (6), SPVD-infected (7) and healthy (8) *I. batatas* TIb 10. Sap dots correspond to approx. 250 μg fresh weight of leaf.

The detection of closterovirus RNA in WTA-infected plants provides evidence that the closterovirus is involved in the sweet potato virus disease complex. Because all of our experiments failed to detect any other agent or its nucleic acid, we believe that the sweet potato virus disease is caused by the interaction between the closterovirus and sweet potato feathery mottle potyvirus. However whitefly transmission studies still have to be done to unequivocally prove that the WTA causing SPVD in mixed infection with SPFMV and the closterovirus are identical.

Closteroviruslike particles were detected, although with some difficulties, in all plants infected with the WTA in single infection. When SPFMV was present—in the SPVD complex—potyvirus particles predominated in leaf dip preparations. Thus, the closterovirus, with a length similar to SPFMV could not be distinguished from the potyvirus in leaf dip preparations. The cDNA clones can be used to examine sweet potato for the presence of the closterovirus—WTA—in either single or in mixed infection; however, only weak signals were obtained in hybridization with extracts from sweet potato infected only with WTA. Future experiments must focus on optimizing the assay by increasing the detection sensitivity with different cDNA clones for detection of viral RNA. Investigations on the distribution of this virus in sweet potato plants will furthermore contribute to the development of an accurate nucleic acid-based detection procedure that allows indexing of sweet potato plants and germ plasm for the incidence of this closterovirus.

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