Survey and Characterization of Viruses in Sweetpotato from Zimbabwe

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ABSTRACT

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Thirty-one clones of sweetpotatoes collected from some parts of Zimbabwe were used as inoculum sources to mechanically inoculate 13 experimental hosts: *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus*, *Datura stramonium*, *Gomphrena globosa*, *Ipomoea purpurea*, *I. quamoclit*, *I. rubrocorulea*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. rustica*, and *N. tabacum*. Systemic vein clearing was observed in *N. benthamiana* inoculated with buffered sap from nine clones. Purification of the vein clearing inducing agent from one of the sweetpotato clones gave yields ranging from 2 to 17 mg/kg and the $A260_{nm}/A280_{nm}$ was around 1.2. Electron microscopy revealed flexuous filamentous particles with a modal length of 830 nm. Protein analysis of purified virus preparations by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed a major protein band of 40 kDa, and this was assumed to be the viral coat protein. Minor protein bands of 27, 37, and 46 kDa were also observed. The viral protein degraded upon storage at 4°C over time to yield a protein band of 27 kDa. Polyclonal antiserum was produced against the purified virus. Protein A gold labeling of the purified virus incubated with available antisera; sweetpotato chlorotic stunt virus (SPCSV), sweetpotato feathery mottle virus strain russet crack (SPFMV-RC), sweetpotato feathery mottle virus, sweetpotato mild mottle virus, sweetpotato latent virus, sweetpotato chlorotic fleck virus, and sweetpotato caulimo-like virus resulted in a higher labeling density with the antiserum of SPFMV-RC than with the antiserum of SPCSV, while the other sera did not react. Further characterization of the vein clearing inducing agent was attempted by reverse transcription-polymerase chain reaction amplification of total RNA with degenerate primers for potyviruses and an oligo dT primer and PCR products of correct size were obtained. The nucleotide sequence was determined and the amino acid of the polyprotein deduced. Comparison with other strains of SPFMV showed strong similarity except for an insertion of 22 amino acids at the N-terminus of the coat protein. The coat protein size of 335 amino acids is the biggest SPFMV so far determined.

Sweetpotato (*Ipomoea batatas* L. Lam) is the third most important root crop after potato and cassava and is ranked seventh in global crop productions (6). The crop is grown exclusively in tropical and subtropical regions with low input and can produce high yield under marginal conditions. In developing countries, sweetpotato is prized by poor farmers as food for human consumption and is often incorporated in multicropping subsistence farming in Zimbabwe. Viral infections have been identified as the second most important biotic component (after insect infestation) in limiting sweetpotato production (13). According to the International Potato Center, crop loss figures in excess of 50% have been attributed to viral infection of sweetpotato. In Zimbabwe, deteriorated crops have been ascribed to viral infection (5). It is known that poor farmers normally retain vine cuttings as "seed" for the next growing season, and this may provide an easy way to perpetuate systemic disease leading to decline in growth and yield potential (22). From a virus disease control perspective, clonal propagation therefore requires a reliable virus identification and detection system (virus indexing scheme) and availability of a virus elimination protocol. Parallel to virus indexing, there is a need to know the full repertoire of the viruses infecting the clonally propagated crop.

Studies indicate that there are five major potyviruses that affect sweetpotato production: sweetpotato feathery mottle virus (SPFMV), sweetpotato mild mottle virus (SPMMV), sweetpotato latent virus (SPLV), sweetpotato vein mosaic virus (SPVMV), and sweetpotato yellow dwarf virus (SPYDV) (18,20). In addition to these potyviruses, other viruses belonging to disparate taxonomic groups have been encountered in sweetpotato virological surveys. These include sweetpotato caulimo-like virus (SPCV); whitefly transmitted closteroviruses, one of which has been designated sweetpotato chlorotic stunt virus (SPCSV) (26); and sweetpotato chlorotic fleck virus (SPCFV isolate C-2), a recently discovered virus of sweetpotato (comparisons with other filamentous viruses of the crop, such as SPFMV, SPMMV, and SPLV, have conclusively shown that it is a distinct virus [3]).

SPFMV is the most thoroughly characterized sweetpotato virus (4) and is known to infect the crop wherever it is grown. Several isolates and strains of SPFMV have been characterized in different parts of the world, perhaps the most important ones being the ordinary (O) (23), russet crack (RC) (20), and severe (S) (17) strains because they directly affect root and tuber quality. SPFMV is frequently identified as a component of synergistic complexes of sweetpotato viruses mostly with whitefly transmitted closteroviruses (8,26) and in most cases interfering with the identification of the other poorly characterized viruses. Although virus elimination protocols based on meristem tip culture have been developed specifically for sweetpotato (15), virus indexing has been hampered by the absence of rapid, sensitive, and reliable diagnostic tools for the viral pathogens (2). Sweetpotato viruses have been detected by observing symptom expression patterns in infected plants in the field or on sensitive indicator plants used in biological assays and occasionally supplemented with confirmatory biochemical assays and electron microscopy. Biological assays of sweetpotato viruses have specific limitations because of coinfection by SPFMV as well as restricted host range (20), low concentration in sap, uneven distribution in the test plants, and possible inhibitors of virus inoculation by plant tissue extracts.

Biochemical assays in sweetpotato virology have been based mainly on immunodiagnostic techniques such as ELISA and filter binding. ELISA has therefore been routinely used to identify specific SPFMV strains and isolates and, to a lesser extent, SPMMV and SPLV (12). While immunodiagnostic techniques are efficient for large-scale testing as in epidemiological studies of sweetpotato viruses (3), they have certain limitations. First, low concentration and uneven tissue-to-tissue dis-

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tribution of sweetpotato viruses reduce their reliability (2). Second, high levels of phenolic compounds, latex, and some uncharacterized inhibitors in sweetpotato tissue extracts also affect reliability and reproducibility of these assays (2).

Thanks to recent advances in molecular biology, rapid, sensitive, and reliable detection of plant viruses based on synthetic nucleic acid probes or the in vitro amplification of specific nucleic acid (DNA) sequences by the polymerase chain reaction (PCR) are now available.

Abad and Moyer (2) developed in vitro transcribed RNA (cRNA) probes (or the so-called "riboprobes"). These probes detected some of the currently recognized SPFMV strains using cDNA containing the 3′-terminal region of the capsid protein cistron, which exhibited a high degree of homology for the potyvirus group. The "riboprobe" system showed greater sensitivity than did immunodiagnostic filter binding, because the riboprobe system overcame interference with host factors that compromise the reliability of immunodiagnostic assays in SPFMV detection.

A PCR protocol has been developed to detect distinct potyviruses in infected sweetpotato (9). Using degenerate primers

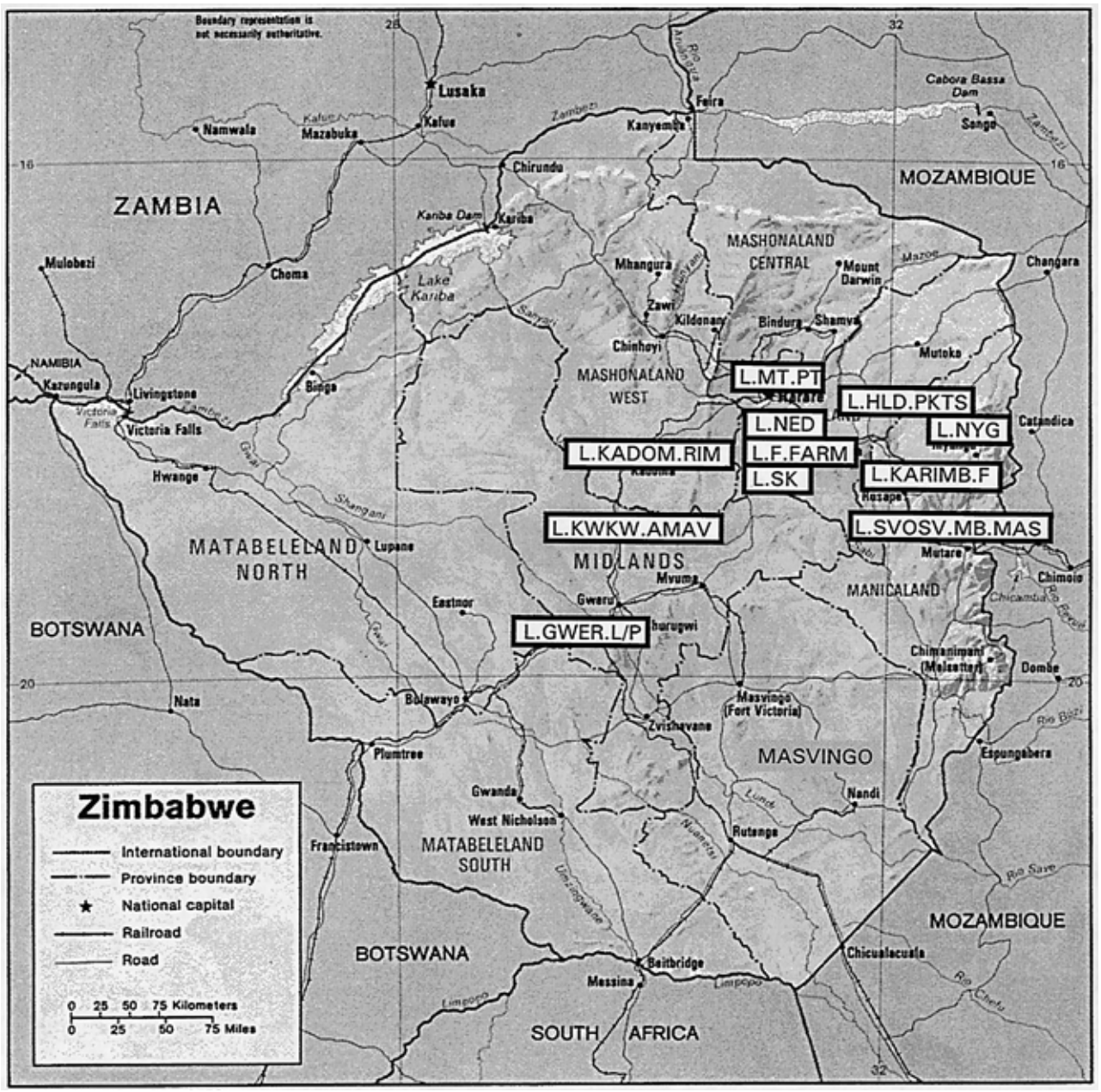

Fig. 1. Location of the collected sweetpotato landraces/clones in Zimbabwe. Description of sample codes: L.F.FARM1,3,6 = clones collected from a site near "Fresh Farm Eggs" kiosk to the South of Harare International Airport, samples 1, 3, and 6; L.GWER.L/P2B,2C,2D = clones collected from Gweru Lower part low density surburb, samples 2B, 2C, and 2D; L.HLD.PKTS1ROB,4ROB,5ROB,6ROB = clones collected from the low density surburb of Highlands at Pocketshill by I. A. Robertson samples 1, 4, 5, and 6; L.KADOM.RIM1B,3A = clones collected from Kadoma in the high density surburb of Rimuka at site 1, sample B and at site 3, sample A; L.KARIMB.F1,3 = clones collected from Karimba Farm, samples 1 and 3; L.KWKW.AMAV1B,2D,3D = clones collected from Kwekwe in the high density surburb of Amaveni at site 1, sample B, at site 2, sample D and at site 3, sample D; L.MT.PT1,2 = clones collected from the low density surburb of Mount Pleasant in Harare, samples 1 and 2; L.NED.GRNT7 = clone collected from a site near Nedlaw (Pvt) Ltd. in the Granitesite area; L.NYG1,4 = clones collected from Nyanga by E. E. Nyamusa samples 1 and 4; L.SK.AN2,3,4,5 = clones collected from Seke Unit A North, samples 2, 3, 4, and 5; L.SK.CN2 = clone collected from Seke Unit C North, sample 2; L.SK.CW8 = clone collected from Seke Unit C West, sample 8; L.SK.GW2,3 = clones collected from Seke Unit G West, samples 2 and 3; L.SK.ME1 = clone collected from Seke Unit M East, sample 1; L.SK.NC1 = clone collected from Seke Unit N Central, sample 1; L.SK.NN1,2,3,4,5 = clones collected from Seke Unit N North, samples 1 to 5; L.SVOSV.MB.MAS2,3,5,7 = clones collected from Svosva at Mbuya Masona's home, samples 2, 3, 5, and 7.

derived from conserved sequence motifs in the genomes of potyviruses designed to amplify the variable 5´-terminal region of the capsid protein cistron, three different potyviruses—a Chinese isolate of SPFMV, SPLV, and a potyvirus closely related to SPFMV—were identified by the amplification of total RNA from sweetpotato clones originating from China. Recently, PCR analysis provided evidence justifying the assignment of SPMMV to the monotypic genus *Ipomovirus* of the Potyviridae (10).

The use of such molecular techniques will certainly improve our knowledge of the epidemiology of sweetpotato viruses and lead to the rationalization of indexing procedures for the screening and certifying of sweetpotato germ plasm repositories as "virus-free." As the full repertoire of sweetpotato viruses is unraveled, the recently developed gene transfer technologies for conferring virus resistance to crop plants (24) may be applied to help reduce the effect of viral infections on sweetpotato productivity.

We report here the first survey undertaken to collect samples from some parts of Zimbabwe that follows up the observation of possible symptoms of sweetpotato virus infection and attempts to identify the virus(es) using experimental host plants. In addition, we isolated and purified a potyvirus from one of the sweetpotato clones and prepared polyclonal antiserum. Viruses infecting the sweetpotato samples were identified by serological methods using available antisera. We also attempted to screen possible potyviruses infecting some of the collected sweetpotato clones using degenerate primers for detection of potyviruses by reverse transcription-PCR (RT-PCR) amplification of total RNA. We were successful in amplifying fragments, and the nucleotide sequence showed high similarity with other strains of SPFMV.

MATERIALS AND METHODS

Origin and maintenance of host sweetpotato plant samples. Sweetpotato landraces were collected as vine cuttings from the field in Zimbabwe at sites depicted in Figure 1. The samples were transferred from Zimbabwe to Wageningen Agricultural University, The Netherlands, as vine cuttings. The vine cuttings were allowed to develop roots in water, planted in pots on a peat medium supplemented with river sand (2:1, v/v), and maintained under greenhouse conditions.

Host-range test. The following indicator host plants were used for mechanical transmission tests: *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus*, *Datura stramonium*, *Gomphrena globosa*, *Ipomoea purpurea*, *I. quamoclit*, *I. rubrocorulea*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. rustica*, and *N. tabacum*. Mechanical transmission was by homogenizing three leaves (corresponding to the youngest, middle, and oldest positions on a vine) of sweetpotato in a chilled mortar containing 15 ml of 0.1 M potassium phosphate buffer, pH 7.2, supplemented with 0.01 M sodium sulfite and rubbing the sap onto leaves of each of the indicator plants previously dusted with Carborundum. The inoculated leaves were subsequently rinsed with running tap water, and the plants were grown in the greenhouse and observed over a period of at least 4 weeks.

After expression of the vein clearing symptom in *N. benthamiana* inoculated with sap from sweetpotato clones L.NED.GRNT7 and L.SK.CN2, the symptomatic tobacco plants were grafted with healthy *I. setosa* plants by slicing parts of the stems of the two plants with sterile blades and tying the damaged parts together with parafilm wrap. Symptoms were monitored over at least 4 weeks.

Virus purification. Clone L.SK.CN2 was chosen as a virus source because it induced vein clearing in *N. benthamiana* during host range testing. These leaves, kept at –20°C, were used to inoculate a single healthy *N. bethamiana* plant. Symptomatic leaves from this single plant then were used as a step-up inoculum for inoculating a suitable number of plants of the propagation host for purifications. Symptomatic plants, 11–20 days post inoculation, were harvested by cutting the stem at about 1 cm above the potting medium surface. This infected tissue (30–270 g) was homogenized at 4°C for 2–4 min in a blender in extraction buffer (0.05 M dipotassium mono-hydrogen phosphate, 0.01 M trisodium ethylene diamine tetra-acetic acid, and 1% [w/v] sodium sulfite, pH 7.6) in variable ratios of 1:1–1:4 (w/v). Ethanol (0.2 ml/g of tissue) was added to the homogenate, which was subjected to 30 s of homogenization. The resultant homogenate was strained through a double layer of cheesecloth and centrifuged for 20 min at 4°C and 16,000 × g_{max} in a Sorvall GSA rotor. Triton X-100 (1%, v/v) was added to the supernatant and the mixture was stirred for 1 h at 4°C. The mixture then was centrifuged for 20 min at 4°C and 16,000 × g_{max} in a Sorvall GSA rotor. The supernatant was collected and centrifuged for 4 h at 4°C and 72,700 × g_{max} in a Beckman rotor Ti45 over a 20% (w/v) sucrose pad made in extraction buffer. Resultant pellets were resuspended in 1 ml of 0.01 M potassium phosphate buffer, pH 7.2. Optical grade cesium chloride (26.7%, w/w) (Gibco BRL) was added to a suitable volume of the suspension and centrifuged for

Table 1. Symptom expression in indicator plants after inoculation with sap from different sweetpotato clones from Zimbabwe

	Indicator plant												
Sweetpotato line	**1[a] *Ca***	**2 *Cq***	**3 *Cs***	**4 *Ds***	**5 *Gg***	**6 *Ip***	**7 *Iq***	**8 *Ir***	**9 *Nb***	**10 *Nc***	**11 *Ng***	**12 *Nr***	**13 *Nt***
L.F.FARM1	LL[b]	LL	–	–	–	–	–	–	VC	–	–	0	–
L.GWER.L/P2C	0	0	–	0	0	0	0	0	VC	SCH,ST	–	0	0
L.GWER.L/P2D	0	0	–	–	0	0	0	0	0	0	0	0	0
L.HLD.PKTS4ROB	0	0	–	–	0	0	0	0	0	M*	0	0	SCH*
L.NED.GRNT7	LL	LL	VC*	0	0	0	CVB	VC,W	VC	VC,ST*	–	0	ST
L.SK.AN2	0	0	–	0	0	0	0	0	VC	0	–	0	0
L.SK.AN5	LL	LL	–	0	0	0	0	0	VC	M*	–	0	SCH*
L.SK.CN2	LL	0	0	–	–	0	0	0	VC	0	–	0	–
L.SK.CW8	0	0	–	0	0	LD	0	0	0	0	–	0	–
L.SK.GW2	0	0	0	–	–	0	0	0	VC	0	–	0	–
L.SK.GW3	0	0	–	0	0	LD	0	VCS	0	–	–	0	–
L.SK.ME1	0	0	–	–	0	LD	0	0	VC	0	0	0	0
L.SK.NC1	LL	0	–	–	–	VC,R	VC,R	–	VC	0	–	0	–
L.SK.NN1	0	0	–	–	0	0	0	0	SCH,ST,N	0	0	0	0
L.SK.NN4	0	0	–	0	0	0	0	0	0	ST	–	0	–
L.SVOSV.MB.MAS5	0	0	0	–	–	0	0	0	VC	0	–	0	–

[a] 1 = *Chenopodium amaranticolor*, 2 = *Chenopodium quinoa*, 3 = *Cucumis sativus*, 4 = *Datura stramonium*, 5 = *Gomphrena globosa*, 6 = *Ipomoea purpurea*, 7 = *Ipomoea quamoclit*, 8 = *Ipomoea rubrocorulea*, 9 = *Nicotiana benthamiana*, 10 = *Nicotiana clevelandii*, 11 = *Nicotiana glutinosa*, 12 = *Nicotiana rustica*, 13 = *Nicotiana tabacum* cv. White Burley.

[b] CS = Chlorotic spots; CVB = chlorotic vein banding; LD = leaf distortion; LL = local lesions; M* = mottle-like; N = necrosis; – = no data; NS = necrotic spots; R = recovery; 0 = symptomless; ST = stunting; SCH = systemic chlorosis; VC = vein clearing; VC* = vein clearing like; VCS = veinal chlorotic spots, W = wilting.

14–16 h at 175,475 × g_{max} and 4°C in a Beckman SW55 rotor. A single opalescent band was observed at about 1 cm from the bottom of the tubes. Bands were pooled and dialyzed against 0.01 M potassium phosphate buffer, pH 7.2, at 4°C with three changes of the dialysis buffer. Virus concentration was determined spectrophotometrically with an extinction of 2.4 at 260 nm for 1 mg/ml solution and a light path of 1 cm.

Estimation of molecular mass of protein. Equal volumes of purified virus preparations, either diluted to suitable concentrations or undiluted, and denaturation buffer (10 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, 0.001% [w/v] bromophenol blue, and 4% [v/v] 2-mercaptoethanol) were mixed and boiled for 2 min, including the markers supplied by Pharmacia consisting of phosphorylase b, bovine serum albumin, ovalbumin, carbonic acid anhydrase, trypsin inhibitor from soybean, and α-lactalbumin with molecular masses of 94, 67, 43, 30, 20.1, and 14.4 kDa, respectively. After cooling to room temperature, sample volumes of up to 10 µl, depending on protein concentration, were applied on to the wells of a 12.5% (w/v) SDS-polyacrylamide gel (14) casted on Mini-PROTEAN II cell components (Bio-Rad) and fractionated by electrophoresis for 40 min at 20 mA, immediately followed by electrophoresis for 1 h at 40 mA. The fractionated gels were either stained with Coomassie Brilliant Blue or used for immunoblotting.

Antiserum preparation. Polyclonal antiserum was prepared from the first virus purification that had a virus concentration of 2.34 mg/ml. After dilution to 100 µg/ml, the virus suspension was emulsified with Freund's incomplete adjuvant (1:1, v/v) and injected into a rabbit intramuscularly. A second (booster) injection was given intramuscularly (400 µg/ml of the same virus preparation as for the first injection) after 3 weeks. The first bleeding was obtained 2 weeks after the booster injection and subsequent bleedings were at 2-week intervals. The serum was designated SPV-Z.

Comparative detection of the purified virus by immunosorbent electron microscopy using different antisera for sweetpotato viruses. Formvar and carbon-coated nickel grids were exposed to a glow discharge before use. Virus particles were adsorbed onto the nickel grids by floating the grids on drops of a virus suspension (0.264 mg/ml) for up to 5 min. After drying, the grids were incubated for 20 min on drops of PBS containing 1% (w/v) bovine serum albumin (PBS-BSA) at room temperature. Polyclonal antisera to SPCSV (obtained from A. A. Brunt), SPFMV-RC (obtained from J. W. Moyer), SPFMV, SPMMV, SPLV, SPCFV, and SPCV (all from M. Querci) were diluted 1:100 and the homologous antiserum were made in PBS-BSA followed by the incubation of the virus adsorbed-grids for up to 45 min. Each antiserum-incubated grid was rinsed with 30 drops of PBS with a 5 min floating of the grid in a drop of PBS. The 5-nm-gold protein A conjugate was diluted in PBS-BSA until a faint pink color was just visible (dilution ratio of about 4 µl of protein A to 300 µl of PBS-BSA). The grids were then incubated in drops of the diluted protein A gold label for up to 45 min followed by rinsing in PBS as before. A fixation step was carried out by floating the grids on drops of PBS containing 1% (w/v) glutaraldehyde for up to 5 min. The grids thus treated were rinsed with double distilled water instead of PBS as described above, and after drying, negative staining was done by floating the grids (including the control) in drops of 2% (w/v) phosphotungstic acid, pH 6.5, for up to 15 s. The grids were viewed with a Zeiss EM109 or Phillips CM electron microscope after drying and photographs were taken.

Detection of oligonucleotide primers by RT-PCR amplification. Sequence

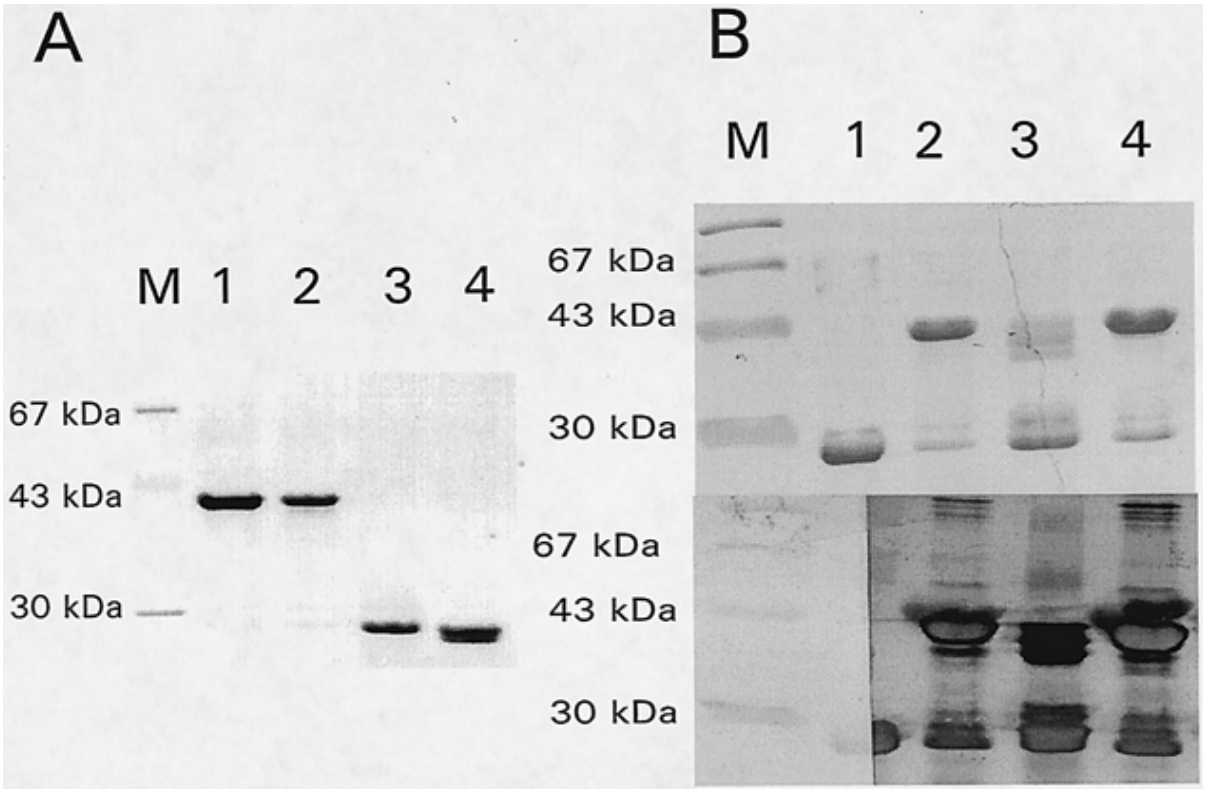

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis to monitor degradation of the putative viral protein(s) after storage from a freshly purified virus preparation at 4°C over time. Numbers on the left are the molecular masses (kDa) of the markers in lanes M: phosphorylase; bovine serum albumin; ovalbumin and carbonic acid anhydrase with decreasing size, respectively. **A,** Effect of storage time. The putative viral capsid protein band migrates are 40 kDa in lane 1 (fresh virus preparation) and 2 (stored 1 week at 4 ℃). This band converted to the 27-kDa protein upon storage for 2 (lane 3) or 3 (lane 4) weeks at 4°C. **B,** Effect of storage temperature. Top panel, two different virus preparations stored for two weeks at 4 ℃ (lane 1 and lane 3) and for two weeks at –20°C. The 27-kDa protein is confirmed as viral degradation product by Western blotting (lower panel) of a co-electrophoresed gel with the same samples.

homology between potyvirus-group specific degenerate primers (Pot1 and Pot2) designed by Colinet and Kummert (9) for the detection of potyviruses in sweetpotato by RT-PCR amplification procedure and the appropriate parts of the available sequences of four strains of the SPFMV was checked using the University of Wisconsin Genetics Computer Group (GCG) sequence analysis program (versions 8 and 9). The degenerate primer sequences were found in all SPFMV strain sequences and therefore ordered from Eurogentec. Also, an oligo dT primer, P89: CGG GAT CCT TTT TTT TTT TTT TTT TTT T, was used for first-strand synthesis and as a pair with Pot2.

For RNA extraction, all glass and plasticware were incubated for 12 h at 120°C. Two methods of RNA extraction from both *N. benthamiana* and sweetpotato were compared—one based on alkaline buffer and sodium dodecylsulfate-phenol-chloroform extraction (method 1) and another based on use of the chaotropic agent, guanidinium isothiocynate (method 2).

Method 1 was as follows: 0.01 g of leaf material was homogenized in 900 µl of extraction buffer (2 vol of 1 M NaCl, 10 mM EDTA, 1 M glycine, pH 9.0, 1 vol of sterile double distilled water, 2 vol of filter-sterilized 10% [w/v] sodium dodecyl sulfate, and 1 vol of 50 mg/ml bentonite) in 1.5 ml Eppendorf tubes. Seven hundred µl of the homogenate was then mixed with 500 µl of phenol and vortexed for 2 min at 40°C with a Thermomixer. After centrifugation at 14,000 × *g* for 5 min, the aqueous phase was mixed with phenol, and the phenol extraction step was repeated twice. The aqueous phase was mixed with 65 µl of 3 M sodium acetate, pH 6.5, and 500 µl of isopropanol and precipitated overnight at –20°C. The precipitated nucleic acids were then pelleted by centrifugation at 14,000 × *g* for 10 min. One ml of 75% (v/v) ethanol was added to each pellet and centrifuged as before. The supernatant was discarded and the pellets dried in vacuo for at least 15 min. The dried pellets were redissolved in 30 µl of sterile double distilled water with vortexing if necessary and stored at –20°C until further use.

Method 2 was essentially as described by Chomcynski and Sacchi (7) except that the leaf material (0.1 g) was directly homogenized in denaturation buffer (4 M guanidium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% [w/v] sarkosyl, and 0.1 M 2-mercaptoethanol) in 1.5-ml Eppendorf tubes with plastic micropestles instead of pulverizing the tissue in liquid nitrogen.

Quality of the nucleic acid extractions was determined by electrophoresis (under RNAse-free conditions) of 5 µl of each preparation mixed with 2 µl of loading buffer (0.25% [w/v] bromophenol blue, 50% [v/v] glycerol, 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA) on a 1% (w/v) agarose gel in 0.5× Tris-borate buffer (TBE) (16) to which ethidium bromide was added (final concentration 0.05 µg/ml). The extracted nucleic acids were visualized by UV-transillumination.

After electrophoretic determination of nucleic acid quality, 10 µl of the extractions were mixed with 2 µl of Pot1 or P89 primer (40 ng/µl, Eurogentec) in PCR Eppendorf tubes and heated to 80°C for 3 min on a Thermal Cycler 480 (Perkin Elmer). The tubes were immediately quenched on ice and the following reagents added: 6 µl of the reverse transcription buffer (5× concentrated, Gibco BRL), 1 µl of dNTP mixture (2.5 mM each, Gibco BRL), 2 µl of dithiothreitol (0.1 M, BRL), 1 µl of RNAsin (40,000 U/µl, Promega), 2 µl of murine moloney leukemia virus reverse transcriptase (0.2 U/µl, Gibco BRL), and 6 µl of double distilled water to give a reaction volume of 30 µl. The tubes were then incubated for 1 h at 37°C on a thermal cycler followed by 10 min of heating at 65°C to denature the enzyme.

The PCR reaction cocktail had a final volume of 40 µl, of which 4 µl was the first strand synthesis product. The other constituents of the mixture included 4 µl of Taq polymerase buffer, (10× concentrated: 0.5 M Tris-HCl, pH 9, 0.5 M KCl, 0.07 M $MgCl_2$, 2 mg/ml BSA), 1 µl of a dNTP mixture (2.5 mM of dATP, dCTP, dGTP, and dTTP), 2 µl each of primer Pot1 and Pot2 (each primer 40 ng/µl, Eurogentec), 2 µl of Taq DNA polymerase (Super Taq, 0.1 U/µl HT Biotechnology Ltd.), and 25 µl of double distilled water. A paraffin overlay of one drop was put in every reaction tube. The thermal cycling profile was as follows: denaturation for 3 min at 94°C followed by five first cycles of denaturation at 94°C for 30 s, annealing at 40°C for 1 min, and extension at 72°C for 2 min, and 31 cycles as for the first five cycles except that annealing temperature was increased to 50°C. Ten microliters of the amplification product was mixed with 3 µl of loading buffer, electrophoresed with 0.5× TBE containing 0.02 µg of ethidium bromide per ml of the electrode buffer in a 1.5% w/v) agarose gel to which 0.05 µg of ethidium bromide had been added per ml of gel solution. Included in the gel electrophoresis was the molecular mass size marker, a PstI digest of lambda DNA. The products were viewed by UV-transillumination and monitored with a Cybertech CS 1 TV camera and photographed.

RESULTS AND DISCUSSION

Host range tests. No symptoms were observed in *D. stramonium*, *G. globosa*, *N. glutinosa*, or *N. rustica*. Mechanical inoculation of the other indicator plants with sap from the sweetpotato clones resulted in two main types of symptoms: local and systemic (Table 1). Chlorotic local lesions were induced in *C. amaranticolor* inoculated with sap from five clones and in *C. quinoa* inoculated with sap from one clone, whereas necrotic local lesions were induced by the inocula from two clones. Inoculum from only one clone (L.NED.GRNT7) induced vein-clearing-like symptoms in *C. sativus*. Also one clone, L.SK.NC1, induced conspicuous vein clearing symptoms in *I. purpurea*, whereas in *I. quamoclit*, sap inocula from L.NED.GRNT7 and L.SK.NC1 caused chlorotic vein banding and vein clearing, respectively. In *I. rubrocorulea*, inoculations with sap from L.NED.GRNT7 (vein clearing) and L.SK.GW3 (veinal chlorotic spots) resulted in systemic infection. Sap inocula from nine clones induced systemic vein clearing in *N. benthamiana* in one assay (during the first 3 months of establishment in the greenhouse), whereas in another (8–10 months later), sap inoculations from only five clones reproduced the same symptoms. In *N. clevelandii*, sap inoculum from L.GWER.L/P2C induced systemic chlorosis, whereas inocula from two clones caused mottle-like symptoms. On *N. clevelandii*, L.NED.GRNT7 and L.SK.NN4 both caused apparent stunting of growth and L.NED.GRNT7 caused

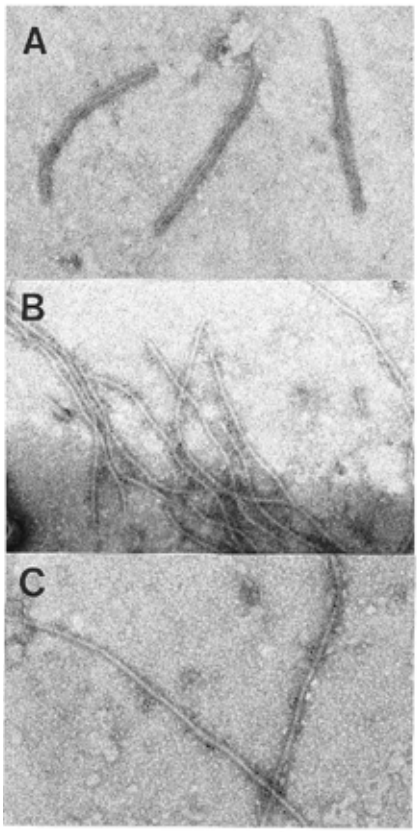

Fig. 3. Electron micrographs of purified vein clearing inducing virus decorated with sweetpotato virus from Zimbabwe (SPV-Z) antiserum **(A),** russet crack strain of sweetpotato feathery mottle virus (SPFMV-RC) antiserum **(B)** and sweetpotato chlorotic stunt (SPCSV) antiserum **(C).** The virus-antibody interaction was probed with 5nm-gold particles to which protein A was adsorbed.

additional vein-clearing-like symptoms. Symptoms similar to systemic chlorosis were observed on *N. tabacum* plants inoculated with sap from L.HLD.PKTS4-ROB and L.SK.AN5, whereas sap from L.NED.GRNT7 induced apparent stunting.

Grafting of vein clearing exhibiting *N. benthamiana* (from previous mechanical inoculations with leaf sap from clones L.NED.GRNT7 and L.SK.CN2) and healthy *I. setosa* plants resulted in systemic expression of the vein clearing symptom followed by a recovery phase.

The assumption was made that SPFMV and whitefly transmitted viruses, notably SPMMV, would infect sweetpotato in Zimbabwe mainly because SPFMV occurs wherever the crop is grown (20). Most of the host range characterizations of different sweetpotato viruses are carried out with a rather restricted range of experimental host plants in the family *Convolvulacea*. Within this family, mainly the *Ipomoea* species such as *I. nil*, *I. purpurea*, and *I. setosa* are used. Many strains of SPFMV have been isolated and studied to varying extents in different parts of the world. The best characterized of these are the RC and C strains. Moyer et al. (19) could differentiate these two strains because the C strain does not infect *I. purpurea* and *Chenopodium* spp. On the basis of the observed reactions in *Chenopodium* spp., the agent causing vein clearing in *N. benthamiana* could therefore be assumed to be SPFMV-RC. However, the RC-strain characteristically infects *I. purpurea*. In this study, only one clone of sweetpotato was shown to have a sap-transmissible agent that infected *I. purpurea* (clone L.SK.NC1). No productive infection was obtained with L.SK.CN2 or the other clones that had local lesion-inducing sap-transmissible agents. This could be a result of the low concentration of the virus in these hosts, which is one of the reasons often cited as to why sweetpotato viruses are difficult to work with (19). Most SPFMV strains described to date do not infect *C. sativus*, *D. stramonium*, *N. clevelandii*, *N. glutinosa*, *N. rustica*, or *N. tabacum*. The RC and C strains do not infect tobacco plants (12,19). However, several strains of SPFMV have been described as infecting *N. benthamiana* in particular, and these include the C1 isolate from Peru (21) and strain 835 from Guatemala (2). Other viruses of sweetpotato, namely SPMMV and SPLV, elicit vein clearing symptoms in *N. benthamiana* but have a wider host range than SPFMV (e.g., they infect *D. stramonium* and *G. globosa*, which are not susceptible to infection by the characterized SPFMV strains) (4).

The development of symptoms observed in *I. setosa* grafted with *N. benthamiana* exhibiting vein clearing from prior inoculation with sap of clones L.NED.GRNT7 and L.SK.CN2 resembled that of SPFMV, suggesting a close relationship of the agent causing vein clearing in *N. benthamiana* with SPFMV.

Overall, the host range data suggest the occurrence of SPFMV strains and not SPMMV and SPLV in the sweetpotatoes from Zimbabwe.

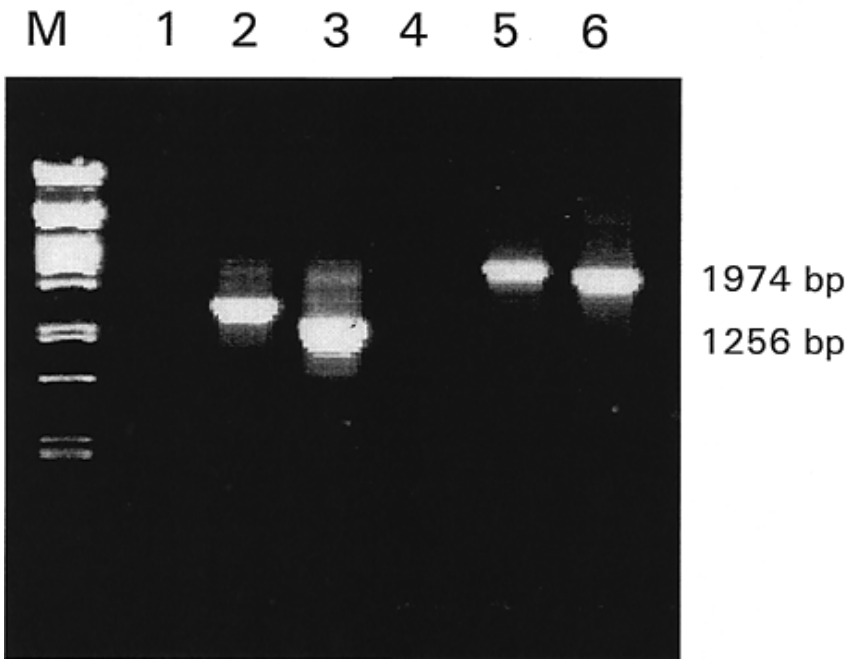

Fig. 4. Agarose gel (1.5 %) electrophoretic analysis of PCR amplified cDNA from: healthy control *N. benthamiana* (lane 1), L.SK.CN2 (lanes 2 and 5) and potato virus^N (lane 3 and 6). RNA was extracted by guanidinium isothiocyanate. Lane 2 and 3: first strand synthesis with Pot1, PCR with Pot2-Pot1 primer pair; lane 5 and 6: first strand synthesis with P89, PCR with Pot2-P89 primer pair. Lane M contains the lambda *Pst*I digest as size marker.

Virus purification and protein analysis. In conjunction with the host range testing, attempts were made to purify a potyvirus from *N. benthamiana* leaves showing vein clearing. The first purified virus preparation was used to raise antiserum. From different purifications, yield ranged from 2 to 17 mg/kg with an uncorrected $A_{260\ nm}/A_{280\ nm}$ of around 1.2. A major protein band with an apparent molecular mass of about 40 kDa was observed in all freshly purified virus suspensions in SDS-PAGE (Fig. 2). Additional bands of apparent molecular masses of 46, 35, 31, and 27 kDa were also observed. After an additional cesium chloride density gradient centrifugation run of some of the purified preparations, the 46-kDa protein band was not detectable by SDS-PAGE and was therefore assumed to be of host origin (data not shown). The other proteins may be an indication of partial degradation of the viral capsid protein due to the purification procedures such as homogenization or host proteolytic activity. Virions of SPFMV-RC and -C strains have a capsid protein with an apparent molecular mass of 38 kDa and as such have atypically large potyvirus capsid proteins. On the basis of capsid protein molecular mass, the purified virus apparently differs from SPFMV-RC/C strains. However, it is remarkable that the putative capsid protein of the purified virus is also atypically large when compared with those of other potyviruses (1).

There was considerable degradation of the protein with storage time (at 4°C) as revealed by SDS-PAGE and immunoblotting (Fig. 2). To prevent the degradation, four different virus purification procedures based on acid precipitation, neutral pH sodium phosphate buffer, *n*-butanol denaturation, and alkaline (borate buffer) pH, respectively, were tested. The expected capsid protein (40 kDa) was present only in the control and the four methods showed numerous protein bands (data not shown). Storage of virus at 4°C quickly degraded the protein and after 2 weeks mainly 27 kDa protein remained (Fig. 2A). Freezing of the purified preparations stopped the degradation to a large extent as shown in Figure 2B with two different virus preparations. Western blot of a co-electrophoresed gel and detection with antiserum raised against the Zimbabwean isolate indicated the viral nature of the degradation bands.

Comparative detection of the purified virus by immunosorbent electron microscopy using different antisera for sweetpotato viruses. Variable degrees of decoration with the protein A-gold label were observed ranging from complete decoration to non-decoration of the flexuous filamentous particles in a purified fraction. The labeling density for the different immunological reactions is shown in Figure 3. Specific and complete decoration of the virus particles by the gold label were shown with the SPV-Z antiserum, whereas

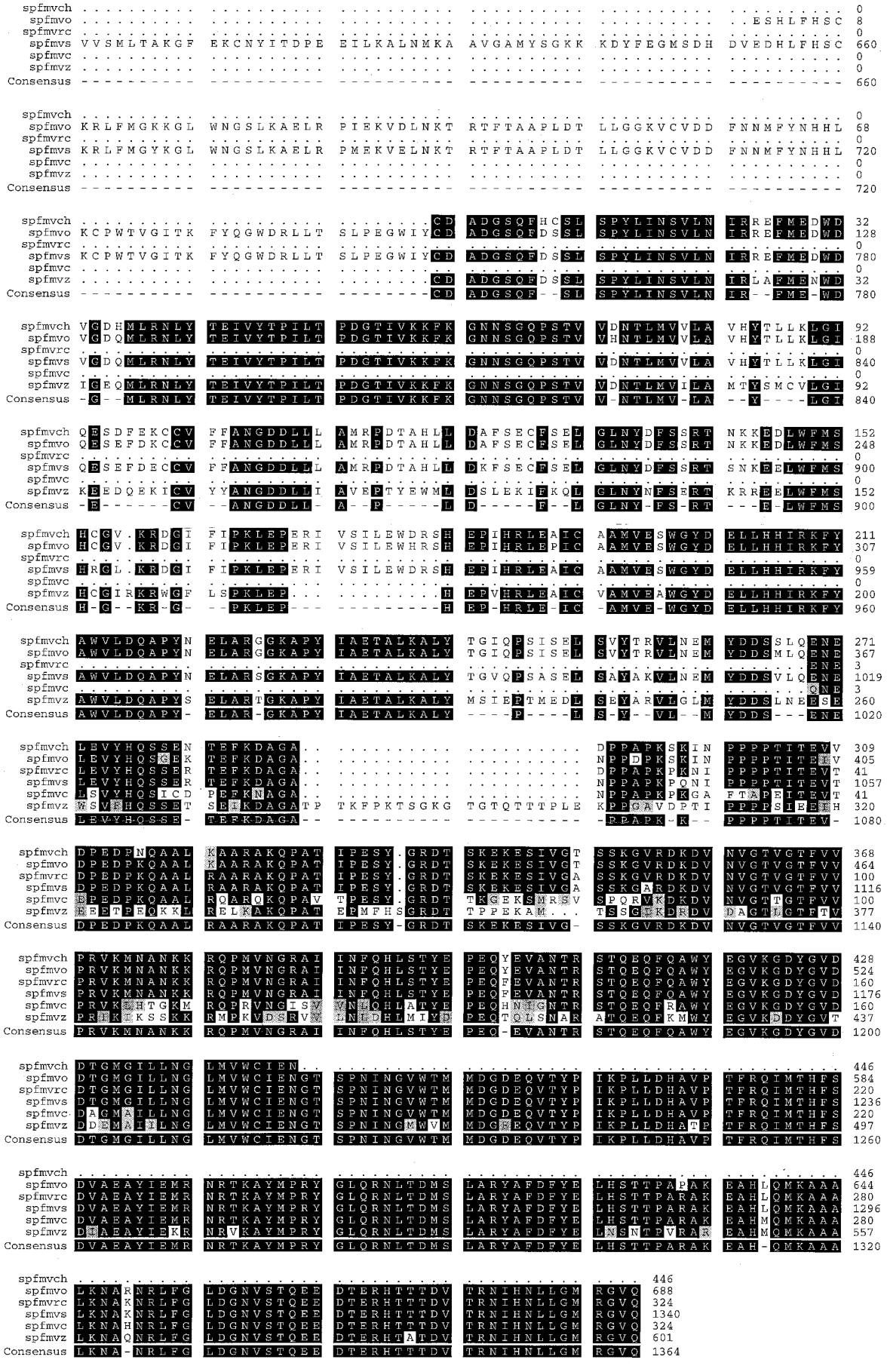

Fig. 5. Comparison of amino acid sequences of the C-terminal part of the polyprotein of strains (C, CH, O, RC and S) of sweetpotato feathery mottle virus together with the SPV-2 (NCBI accession number AF016366) from Zimbabwe and the consensus sequence.

significant decoration was also seen in the virus preparation that reacted with the antiserum for SPFMV-RC. The distribution of the protein A-gold label was patchy in the virus preparation incubated with antiserum for SPCSV and, in the case of the antiserum for SPFMV, there was hardly any detectable label. There was no decoration of the virus particles reacted with antisera for SPMMV, SPLV, SPCFV, or SPCV.

The data showed qualitative correlation with that obtained by the dot-blot immunoassays in terms of reactivity of the homologous antiserum and the antisera for SPFMV-RC, SPCSV, and SPFMV with the purified virus. Using an essentially similar technique of protein A-gold labeling, Usugi et al. (23) also demonstrated varying degrees of decoration of particles of three filamentous viruses of sweetpotato in homologous or heterologous combinations. These viruses had until then been considered to be SPFMV. Protein A-gold labeling of antiserum decorated virus preparations has been shown to be a very sensitive tool capable of differentiating distantly related viruses though strains of the same virus cannot be distinguished (25). Therefore, the data from immunogold protein A staining with antisera for SPFMV-RC and SPCSV suggest that SPV-Z shares common epitopes particularly with SPFMV-RC more than with SPCSV. This result also suggests cross-reactivity of the antisera for SPFMV-RC (a potyvirus) and SPCSV (a possible closterovirus), and that is reported here for the first time.

Detection by RT-PCR amplification. Each of the two nucleic acid extraction procedures yielded intact ribosomal RNA from the leaves of either sweetpotato or vein-clearing-exhibiting *N. benthamiana*. In addition to that, all extractions by the SDS-phenol-chloroform method yielded high-molecular-weight DNA from the host plant. Thus, the nucleic acid extractions were considered to be suitable for use in the RT-PCR assay. The expected products (1.3 kb for Pot1 and Pot2, and 2.0 kb for P89 and Pot2) were detected in the positive control, PVY^N. The Zimbabwean isolate amplified products that were slightly bigger (Fig. 4).

The data show that the degenerate primers designed from conserved regions of the potyvirus genome for the detection of sweetpotato viruses did detect the virus isolated and purified from a Zimbabwean sweetpotato clone, which showed close serological relationships with a well-characterized strain of SPFMV, the russet crack strain.

The obtained fragments were cloned and sequenced and the amino acid sequence was compared with other strains of SPFMV (Fig. 5). The remarkable size of the Zimbawean isolate was confirmed. The increase to 335 amino acids compared to the other strains of SPFMV was largely accounted for by the 22 amino acid insert at the N-terminus of the coat protein.

In conclusion, the reported work demonstrated the occurrence of a mechanically transmissible virus of sweetpotatoes from Zimbabwe that induced vein clearing in *N. benthamiana* and from which the virus was purified to produce polyclonal antiserum. Comparative detection of the virus with available antisera using immunogold labeling electron microscopy showed that the purified virus shared common epitopes particularly with SPFMV-RC and therefore has a close serological relationship with SPFMV-RC. Future cloning and sequencing of the viral genome will reveal its position within the potyviruses and allow the development of a sensitive, fast, and reliable PCR test to monitor the development of this virus in fields with new virus-free sweetpotato clones.

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