Original Research Article

Biological, serological and molecular characterisation of a new virus species infecting *Telfairia occidentalis* in Calabar, Cross River State, Nigeria

ABSTRACT

Telfairia occidentalis (Hook) belongs to the family Cucurbitaceae and is an economically important cash crop worldwide. It is widely cultivated in Nigeria including the South Eastern part of the country. This research was aimed at isolating, characterising and identifying a new virus species infecting *Telfairia occidentalis* in Calabar, Cross River State. Diagnostic tools employed included host range/symptomatology, insect transmission test, Antigen Coated Plate (ACP) Enzyme Linked-Immunosorbent Assay (ELISA) and gene sequencing. Results revealed that the virus isolate infected only members of the cucurbit family producing rugosity, mosaic, mottle and leaf malformation/deformation. The virus isolate was transmitted by *Aphis spiraecola* in a fore-gut manner and not by *A. citricida*. It reacted positively against universal potyvirus antiserum. Sequence analysis showed that the *Telfairia occidentalis* virus isolate had 75 % sequence identity with *Moroccan watermelon mosaic virus* (MWM) which was found to be the closest. The virus was consequently considered a new species of potyvirus for which the name Telfairia severe mosaic virus (TeSMV) was suggested.

Keywords: *Telfairia occidentalis*, ACP-ELISA, Potyvirus, *Telfairia severe mosaic virus.*

Introduction

Telfairia occidentalis is a tropical vine grown in West Africa as a leaf vegetable and for its edible seeds. It is a drought-tolerant, dioecious and perennial crop that is usually grown trellised. Common names for the plant includes fluted gourd, fluted pumpkin, ugu (in the Igbo language), and ikong-ubong (in the Efik/Ibibio language). *Telfairia occidentalis* is a member of the Cucurbitaceae family and is indigenous to southern Nigeria [1]. The leaves of *T. occidentalis* are rich in protein (4.30), fat (1.80), fibre (2.30) and ash (6.10 g/100 g) [2]. The seeds produced by the gourd are also high in protein and fat, and can, therefore, contribute to a well-balanced diet. The aqueous extract has been reported to have a high content of protein and iron which is useful in the treatment of anemia due to the hematinic properties of the leaf [3]. A recurring subject in the Igbo's folklore, the fluted gourd is noted to have healing properties and was used as a blood tonic, to be administered to the weak or ill. It is endemic to southern Nigeria, and was an asset to international food trades of the Igbo ethnic group [1].

The vegetable is traditionally used by an estimated 30 to 35 million indigenous people in Nigeria, including the Efik, Ibibio, and Urhobo [1]. In the south eastern states of Nigeria, it is considered the foremost among the leafy vegetables [2].

This crop has been reported to be threatened by diseases of fungal, bacterial and viral origin [4] and pests, such as the pumpkin caterpillars (*Diaphania* spp.) and the melon fly (*Bactrocera cucurbitae* Coquillet), are also serious constraints to their cultivation [5]; [6]; [7]; [8]. Aphids (*Aphid spiraecola*) whiteflies (*Bemisia tabaci*) and thrips (*Thrips abyssiniae*) can become serious limitations to production of this crop through the extensive damage they

provoke and also through the transmission of viral diseases [9]; [10]; [11]. Plant viruses are ranked the most common causal agent of cucurbit diseases worldwide [12].

A visit to gardens and farms in the University of Calabar staff quarters during 2015 planting season revealed widespread infection of the crop. Infected plants exhibited virus-like symptoms such as mosaic and mottling. This study was therefore aimed at employing serological and molecular tools in characterising the virus responsible for the infection of this crop with a view to identifying it.

Materials and methods

Sources and isolation of viruses

Telfairia occidentalis leaves showing typical virus-like symptoms were obtained from the staff quarters of University of Calabar, Cross River State Nigeria. Infected leaves samples were collected into Ziploc® air tight polyethylene bags to keep the leaves fresh to ensure the viability of the viruses.

Preparation of virus inocula

Virus inocula were prepared by triturating symptomatic infected leaves tissues of the samples in pre-sterilized cold pestle and mortar in the inoculation buffer.

Buffer preparation

The buffer used for inoculation in this study was 0.03 M disodium hydrogen orthophosphate buffer ($Na_2H_2PO_4.2H_20$). pH 8.0. 4.26 was dissolved of disodium hydrogen orthophosphate in one litre of distilled water. The pH was adjusted by adding few drops of 0.5 M sodium dihydrogen phosphate (NaH_2PO_4) to obtain the desired pH 8.0 and was kept cold before use.

Host range studies

Seeds used for host range studies were obtained from local farmers in Okurikang (Odukpani Local Government Area), Adim (Biase L.G.A.), Butatong (Boki L.G.A.) and Watt market,

Calabar, Nigeria. For the host range studies, 35 plant species belonging to 12 families tested were: *Chenopodium amaranticolor, Trichosanthes cucumerina, Citrullus lanatus, Cucumis melo, Cucumis sativus, Cucurbita pepo, Luffa cylindrica, Nicotiana tabacum, Lycopersicon esculentum, Capsicum annuum, Phaseolus vulgaris, Telfaira occidentalis, Cucumeropsis manni, Cucurbita moschata. Cucurbita maxima, Lagenaria siceraria, Arachis hypogaea,, Phaseolus vulgaris, Amaranthus hybridus, Solanum melongena, Ocimum gratissimum, Sorghum bicolor, Abelmoschus esculentus, Oryza sativa, Nicotiana glutinosa, Nicotiana rustica, Datura stramonium, Helianthus annuus, Chenopodium album, Vigna sinensis, Vigna mungo , Vigna radiata, Dolichos lablab, Amaranthus viridis, and Gomphrena globosa*. The seeds were sown in 16 cm diameter polyethylene bags, ¾ filled with sterilized humus soil at the rate of five seeds per bag. Inocula prepared from the symptomatic leaf tissues were inoculated mechanically on carborundum (600 mesh) dusted leaves of the test plants. The cucurbits and fabaceous plants were inoculated at 2 leaf stage and others at 4-5 leaf stage. Inoculated leaves were then rinsed with water and left for symptoms development.

Insect transmission tests7

The ability of *Aphis spiraecola* and *A. citricida* to transmit the virus used in this study was tested by obtaining the aphids from their natural host *Chromolaena odorata* and *Citrus sinensis* respectively. Insects were collected in a transparent plastic container over which a piece of gauze was firmly secured. After the insects were starved for one hour and then allowed acquisition access feeding period of 2-5 minutes on virus infected leaves. A paint brush was used to transfer 5 aphids to each of the ten polyethylene pot of containing young seedlings of *Cucumeropsis manni,* (The test plant) inside insect proof screen cages for inoculation access feeding of 10 minutes. The aphids were killed by spraying them with aphicide (Pirimor). This experiment was carried out once

Serological tests

Antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA) as described by [13] was used to determine the genus to which the virus belonged. Symptomatic leaf samples of 0.1 g were triturated in 1 mL of coating buffer (0.015 M Na_2CO_3 + 0.0349 M $NaHCO_3$ + dH_2O) and dispensed into each well of ELISA plate. After incubation at 37°C for 1 h the plate was washed 3 times with PBS-Tween for 3 min between each wash. Cross adsorption was made by grinding 1 g of healthy plant sample in 20 mL of conjugate buffer ($^1/2$ PBS + 0.05% Tween 20 + 0.02% egg albumin + 0.2% PVP). Antisera to CMV and the universal potyvirus antiserum were diluted at 1:3000 in the adsorption solution and 100 µL of each antiserum polyclonal antisera were added to wells of the ELISA plates and again incubated at 37°C for 1 h. The ELISA plates were then washed 3 times with PBS-Tween. One hundred-µL of protein, A-alkaline phosphatase conjugate diluted in the ratio 1:15000 in conjugate buffer ($^1/2$ PBS + 0.05% Tween 20 + 0.02% egg albumin + 0.2% PVP + 0.02 g $NaNO_3$) was added per well and the plates incubated at 37°C for 1 h. The plates were again washed 3 times with PBS-Tween. One hundred-µL of 0.001g mL^{-1} of *p*-nitrophenyl phosphate substrate in substrate buffer (97 mL diethanolamine + 800 mL H_2O + 0.2g $NaNO_3$ and HCl to give pH 9.8) was added per well and incubated at room temperature for 1 h. For all incubations, plates were covered with ELISA cover plates to avoid edge effects and to maintain uniform temperature. Healthy plant sample was used as controls. After 1 h absorbance was measured at $A_{405\ nm}$ using an ELISA plate reader (Micro Read 1000 ELISA Plate Analyser, U.S.A). After 1 h of incubation the samples were considered positive when the ELISA reading was at least twice the reading for the healthy control [13].

RNA extraction from infected leaf samples

Total RNA was extracted from the infected leaf samples using the cetyltrimethylammonium bromide (CTAB) protocol as described by [14]. One hundred milligrams of each infected leaf sample was grounded in sterile mortar and pestle in 1 ml extraction buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2 % CTAB) (hexadecyltrimethylammonium bromide); and 0.4 % β- mercaptoethanol, added just before use. Each of the homogenates was poured into a new 1.5 mL Eppendorf tube. The tubes were vortexed briefly, incubated in a 60^{o}C water bath for 10 minutes and allowed to cool to room temperature. Then 0.75 mL of phenol chloroform isoamyl (25:24:1) was added to each tube containing the homogenate. Each tube was then vortexed vigorously to form an emulsion and then centrifuged at the speed of 12,000 RCF for 10 minutes. The supernatant was then transferred to a clean 1.5 mL tube. It was added 300 mL of cold isopropanol to the supernatant to precipitate the nucleic acid (RNA) and the mixture was kept at -80^{o}C for 10 min. The mixture was centrifuged at 12,000 RCF for 10 min to precipitate the nucleic acid. The supernatant was discarded and the nucleic acid pellet washed in 500 µL of 70 % ethanol and centrifuged at 12,000 RCF for 5-10 minutes. The supernatant was decanted and the resultant nucleic acid pellet was air-dried at room temperature. Nucleic acid pellet was then re-suspended in 50 µL sterile distilled water and used as a template source for reversed transcriptase polymerase chain reaction (RT-PCR). Nucleic acid extracts from the leaves of healthy plants were used as negative control.

Reverse Transcriptase Polymerase Chain reaction (RT-PCR)

Virus-specific complementary DNA (cDNA) fragments were amplified from total nucleic acid derived from the infected leaf samples by a RT-PCR method as described by [15]. RT-PCR was performed using the cylindrical inclusion (CI) primers Forward 5'-GGIVVIGTIGGIWSIAARTCIAC-3', Reverse 5'-ACICCRTTYTCDATDATRTTIGTIGC-3' and CMV primers Forward 5'-TGGTCGTCCAACTATTAACCAC-3' Reverse 5'-TACTGATAAACCAGTACCGGTGA-3' as described by [16]. The RT-PCR reaction

mixture (50 µL) consisted of I µL each of CICP 5' and CICP 3', 5x Go Taq green buffer (10.0 µL), $MgCl_2$ (3.0), dNTPs (1.0 µL), Reverse transcriptase (0.24 µL), Taq DNA polymerase (Promega) (0.24 µL), sterile distilled water (30.52 µL) and nucleic acid from infected sample (1:10 dilution) (3.0 µL).

Amplifications were carried out in a GeneAmp 9700 PCR System Thermal Cycler (Applied Biosystem Inc., USA) using the following thermocyclic conditions; 42° C for 30 min for reverse transcription, 94°C for 3 min for initial denaturing, followed by 40 cycles of denaturing at 94° C for 30 sec, an annealing step at 40° C for 30 s, an extension at 68°C for 1 min and a final extension at 72°C for 10 min ended the RT-PCR reaction. The PCR reaction products were separated on 1.5 % agarose gel, subsequently stained with ethidium bromide, visualized in UV light and photographed RT-PCR assay produced a PCR amplicon of expected size (approximately 700 bp for potyvirus and 500 bp for CMV).

Amplicon purification and sequencing

The RT-PCR amplicon for each sample was purified by adding 95 % ethanol to 40 µL of the amplicon in a new 1500 µL Eppendorf tube and the solution was kept at – 80° C for 10 minutes. The tube was centrifuged for 10 min and the supernatant discarded. It was added 50 µL of 70 % ethanol and centrifuged at maximum speed for 5 min. The supernatant was discarded and the tube was left at room temperature to dry after which the purified cDNA was dissolved in 30 µL of sterile distilled water. The product was sequenced at Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria.

Sequence analysis

The sequence identities between the viruses under study were established by comparison with known virus sequences in the GenBank available at National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) program [17].

Sequence identities were calculated from the "sequence identity matrix" option in MEGA 6 Windowsoftware, Phylogenic trees were constructed from the muscle-aligned sequence using MEGA version 6 [18] using a neighbor-joining method. Parameter model for estimating the distance and conducting the bootstrap analysis and evolutionary divergence were estimated using MEGA version 4 [19].

Results

Host range study and symptomatology

All members of the cucurbit family used in this study were susceptible to the virus isolates. Symptoms induced ranged from mosaic, mottling, leaf malformation/deformation, rugosity to chlorotic spots. The virus isolate induced mosaic symptoms on *Telfairia occidentalis, Cucurbita moschata* and *Cucumis melo.* It also induced mottling on *Cucumis sativus,* and *Lagenaria siceraria. Mosaic* and *mottling* were induced on *Cucurbita pepo, mottling* and rugosity on *Cucumeropsis manni,* leaf reduction and rugosity on *Trichosanthes cucumerina,* chlorotic spots on *Citrillus lanatus* and rugosity/leaf malformation and deformation on *Luffa cylindrical* (Table 1). Plant species belonging to other plant families were not susceptible to the virus isolate.

Insect transmission tests

Results obtained from this study showed that *A. spiraecola* successfully transmitted the virus isolate while *A. citricida* did not transmit the virus (Table 2).

Serology testing against potyvirus and CMV antisera

The results of serological testing showed that the virus isolate reacted positively against the universal potyvirus antiserum, but there was no reaction against the CMV antiserum (Table 3)

Gene sequence and sequence alignment

Figure 10 represents nucleotide sequence for the Telfairia virus isolate while Figure 11 represents the gene alignment of the sequence when compared to other viruses available in the GenBank. The sequence alignment revealed 75 % sequence homology with *Moroccan watermelon mosaic virus* when compared to other virus sequences using BLAST program.

TABLE 1

Reaction of host plants to sap inoculation of virus isolate

Plant species	Symptoms
1. *Telfairia occidentalis*	LMD, C
2. *Cucumis sativus*	M
3. *Cucumeropsis manni*	R, Mo
4. *Cucurbita pepo*	M
5. *Cucurbita moschata*	M
6. *Luffa cylindrical*	R, LMD
7. *Citrillus lanatus*	C.S
8. *Lagenaria siceraria*	Mo
9. *Trichosanthes cucumerina*	LR, R

CS=Chlorotic spots; M=Mosaic; Mo=Mottling; LMD=leaf malformation/deformation; R= Rugosity and LR- Leaf reduction

Figure 1. Leaf malformation/deformation and mosaic on *Telfairia occidentalis*

Figure 2. Mosaic symptom on *Cucumis sativus*

Figure 3. Rugosity and severe leaf malformation/deformation on *Luffa cylindrical*

Figure 4. Mottling on *Lagenaria siceraria*

Figure 5. Mosaic symptom on *Cucurbita pepo*

Figure 6. Mottle and rugosity on *Cucumeropsis mannii*

Figure 7. Severe leaf reduction and rugosity on *Trichosanthes cucumerina*

Figure 8. Mosaic symptom on *Cucurbita moschata*

Figure 9: *Citrullus lanatus* showing chlorotic spots

TABLE 2

Insect transmission test

Virus isolates	Aphid species	% Transmission
Telfairia virus isolate	*Aphis spiraecola*	100
	Aphis citricida	0

TABLE 3

Antigen Coated Plate (ACP) Enzyme-Linked Immunosorbent Assay (ELISA) for detection of CMV and Potyviruses

Sample	Location	OD reading at A_{405nm} against Virus Polyclonal Antibodies	
		CMV	Potyvirus
Telfairia virus isolate	Calabar	0.529	1.105*
Healthy Control		0.326	0.405
Infected Control		2.687	1.894

***Sample were considered virus positive when the optical density (OD) reading at *A*405 nm was 2x greater than the absorbance from healthy controls**


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TCGGCGGTGGGGTGGGGGAAGTCGACTGTAACCCCCACCTCTTGGAAGGGGGGGGAGGGGGAGAAATCTCCTTATTGAACCAA
CTAAACCTTTGTGTGAGAATGTCTGCAAGCAACTCAGAAGTGATCCATTCCACAAGAACCCAACAATTCGCATGAGAGGACTA
ACTTCCTTCGGTTCAACGCCAATTACCATCATGACTAGCGGATTTGCCTTGCATTACTTTGCGCACAATACAGAACAACTTAG
GGAATTCGATTTTGTTATATTTGATGAATGCCATGTGGTTGATGCTCAGGCTATGGCGTTCTACAGCTTACTCGAGGGTTATG
CCATCAAAACAAAAATTCTCAAAGTGTCAGCAACGCCACCTGGGCGTGAAACAGATTTCTCAACACAACATCCTGTTAAAATT
GTGACTGAGAATTCTATTAGCTTCCAACAACTGGTTGCAAGCTTTGGCACTGGTTCCACTAGTGACGTGACAGCGAAAGCTGA
CAACATACTAGTTTATGTTGCAAGTTATAATGAAGTTGATCAATTGAGTAAATTACTCAATGAGAGAGGATATCTAGTCACGA
AAGTGGATGGGAGAACAATGAAAGTTGGACGCACTGAAATAGAATCGCGAGGAACTCCACAGAAGAAACACTTCATTGTTGCC
ACCAACATCATCGAAAACGGGCGTAGA
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Figure 10: Gene sequence for Telfairia virus isolate

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Query  68    CTCCTTATTGAACCAACTAAACCTTTGTGTGAGAATGTCTGCAAGCAACTCAGAAGTGAT  127
             || || |||||||||||  | |||  |||||||||||||  || |||||  || |||  ||||
Sbjct  4069  CTTCTCATTGAACCAACAAGACCACTGTGTGAGAATGTATGTAAGCAGCTTAGAGGTGAA  4128

Query  128   CCATTCCACAAGAACCCAACAATTCGCATGAGAGGACTAACTTCCTTCGGTTCAACGCCA  187
             |||||  ||||||||||||||||  ||  | ||||||||  ||  |||||  ||  ||||||||  |||
Sbjct  4129  CCATTTCACAAGAACCCAACTATCAGAATGAGAGGTCTCACTTCATTTGGTTCAACTCCA  4188

Query  188   ATTACCATCATGACTAGCGGATTTGCCTTGCATTACTTTGCGCAC--AATACAGAACAAC  245
             |||||  | |||||  ||  ||  ||  ||  |||||  |  |||  |||||  |||  |||  ||||
Sbjct  4189  ATTACAGTAATGACAAGTGGTTTCGCTTTGCACT--TTTACGCACATAATGTAGAGCAAC  4246

Query  246   TTAGGGAATTCGATTTTGTTATATTTGATGAATGCCATGTGGTTGATGCTCAGGCTATGG  305
             |    ||||  |||  ||  |  ||  ||||||||||||||  ||  ||||  ||  |||||  ||||
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Sbjct  4247  TATCTGAATACGACTTCATCATTTTTGATGAATGCCACGTAATTGACGCGCAGGCAATGG  4306

Query  306   CGTTCTACAGCTTACTCGAGGGTTATGCCATCAAAACAAAAATTCTCAAAGTGTCAGCAA  365
              ||||||  |  || |  |||   ||  ||| |||   ||  | |||||||| || ||||
Sbjct  4307  GGTTCTATTGTCTAATGAAGGAGCATAGCATTAAAGGGAAGGTCCTCAAAGTTTCTGCAA  4366

Query  366   CGCCACCTGGGCGTGAAACAGATTTCTCAACACAACATCCTGTTAAAATTGTGACTGAGA  425
              |||| |||||  | |||||||| || ||||| ||||| || || ||| | || || |||
Sbjct  4367  CGCCTCCTGGAAGGGAAACAGAATTTTCAACGCAACACCCAGTCAAATTAGTCACGGAGG  4426
Query  426   ATTCTATTAGCTTCCAACAACTGGTTGCAAGCTTTGGCACTGGTTCCACTAGTGACGTGA  485
              |    || ||||| |||||| |||| |||| ||||||||||||  | | |||||| || |
Sbjct  4427  AAAGCATCAGCTTTCAACAATTGGTAGCAAACTTTGGCACTGGAGCGAATAGTGATGTCA  4486
Query  486   CAGCGAAAGCTGACAACATACTAGTTTATGTTGCAAGTTATAATGAAGTTGATCAATTGA  545
              |   |   ||| |||||||||| || ||||| || || || ||||||||||||||| |
Sbjct  4487  CCAAGTGCGCTAACAACATACTCGTGTATGTCGCTAGCTACAATGAAGTTGATCAACTTG  4546
Query  546   GTAAATTACTCAATGAGAGAGGATATCTAGTCACGAAAGTGGATGGGAGAACAATGAAAG  605
              | ||  |  |   ||| | ||| || || |||||||||||||||| ||||| |||||||
Sbjct  4547  GAAAGCTTTTGCTTGATAAAGGGTACCTTGTCACGAAAGTGGATGGAAGAACGATGAAAG  4606

Query  606   TTGGACGCACTGAAATAGAATCGCGAGGAACTCCACAG-AAGAAACACTTCATTGTTGCC  664
              ||||| | || || |||||| ||  ||||||    ||| |||||||| ||||||||||
Sbjct  4607  TTGGAAGAACAGAGATAGAAACGAAAGGAAC-AAGCAGCAAGAAACATTTCATTGTTGCA  4665

Query  665   ACCAACATCATCGAAAACGG  684
              |||||||| ||||| || ||
Sbjct  4666  ACCAACATTATCGAGAATGG  4685
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Figure 11. Gene alignment of Telfairia virus isolate showing 75 % sequence homologue with *Moroccan watermelon mosaic virus* isolate

Discussion and Conclusion

The result of the host range and symptomatology studies indicated that the virus isolate had a rather narrow host range causing systemic infection on all tested cucurbits with symptoms ranging from mosaic to severe leaf malformation/deformation. No symptoms were observed on other plant species tested.

Reports have shown that members of the genus *Potyvirus* most commonly have narrow host ranges limited to the cucurbit family [20]; [21]; [22]; [23]; [24]; [25]; [26]; [27], suggesting that the virus isolates in this study could belong to the genus *Potyvirus*.

The virus isolate in this study was transmitted in a non persistent manner by *A. spiraecola*. *A. citricida* did not transmit the virus. Several reports on *Aphid spiraecola* transmitting potyviruses have been documented, this result is therefore consistent with such report that *Aphid spiraecola* transmit viruses in the genus *Potyvirus* in a non persistent manner.

Similar observations have been reported for members of this genus and these viruses could obviously belong to the genus *Potyvirus* [28]; [24]; [29]; [30]; [31] [32].

ACP-ELISA has been employed in the detection and identification of plant viruses into the genus taxon [33]; [34]; [35]; [36]; [37]; [38]; [39]. The detection of the virus isolate in this study by universal potyvirus antiserum further suggests it belong to the genus *Potyvirus.*

Gene sequencing as tool for virus identification and characterization has become the ultimate in recent times [29]; [40] [41]; [30]; [42]; [28]; [43]; [21]; [26]; [44]; [45].

A virus identity will become unassailable if the degree of homologue of it sequence is established after comparison with sequences of previously characterised members of the genus to which the virus in question belongs.

It has been suggested by [46]; [47]; [48] that virus sequence with less than approximately 76 % nucleotide sequence identity should be regarded as belonging to different species while isolates with 76-89 % sequence identity should be considered as virus of the same strains and sequence presenting 90-100 % sequence identity should be regarded as belonging to the same virus species.

The virus isolated from *Telfairia occidentalis* in the University of Calabar Staff Quarters in this study had 75 % sequence identity falling below the thresh hold of 76 % and was thus considered a different species of virus and Telfairia severe mosaic virus was suggested as the name for the new virus.

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