

Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda

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The molecular variability of cassava geminiviruses occurring in Uganda was investigated in this study. Infected cassava plants and whiteflies were collected from cassava plantings in different geographical areas of the country and PCR was used for molecular characterization of the viruses. Two complete sequences of DNA-A and -B from *African cassava mosaic virus* (ACMV), two DNA-A sequences from *East African cassava mosaic virus* (EACMV), two DNA-B sequences of EACMV and the partial DNA-A nucleotide sequence of a new virus strain isolated in Uganda, EACMV-UG3, are reported here. Analysis of naturally infected cassava plants showed various assortments of DNA-A and DNA-B of the Ugandan viruses, suggesting the occurrence of natural inter- and intraspecies pseudorecombinations and a pattern of cassava mosaic disease (CMD) more complex than previously reported. EACMV-UG2 DNA-A, which contains a recombinant fragment between ACMV and EACMV-UG1 in the coat protein gene that resembles virus from Tanzania, was widespread in the country and always associated with EACMV-UG3 DNA-B, which probably resulted from another natural recombination event. Mixed infections of ACMV-UG and EACMV-UG in cassava and whiteflies were detected in most of the regions where both viruses occurred. These mixed-infected samples always showed extremely severe CMD symptoms, suggesting a synergistic interaction between ACMV-UG and EACMV-UG2. The first demonstration is provided of infectivity of EACMV clones to cassava, proving conclusively that the pseudorecombinant EACMV-UG2 DNA-A+EACMV-UG3 DNA-B is a causal agent of CMD in Uganda.

Introduction

Cassava mosaic disease (CMD) is the most important disease of cassava in Africa, causing an estimated loss of yield of over 1.5 billion US dollars a year (Thresh *et al.*, 1994). Several whitefly-transmitted viruses belonging to the genus *Begomovirus*, family *Geminiviridae*, cause the disease. The majority of whitefly-transmitted geminiviruses have bipartite genomes with A and B components. The two components

share only a 'common region' (CR) of approximately 200 bp with high sequence identity (90–100%). The CR contains promoter and sequence elements required for DNA replication and transcription (Chatterji *et al.*, 1999; Eagle *et al.*, 1994; Laufs *et al.*, 1995; Zhan *et al.*, 1991). Component A encodes all viral proteins necessary for replication and encapsidation of both DNAs (Rogers *et al.*, 1986; Stanley, 1983; Sunter *et al.*, 1987; Townsend *et al.*, 1986) and the B component encodes two proteins necessary for efficient systemic spread of the virus throughout the plant (Brough *et al.*, 1988; Etessami *et al.*, 1988; Ingham *et al.*, 1995; Von Arnim *et al.*, 1993).

CMD has been reported in all cassava-growing countries in Africa and the Indian subcontinent. Three separate virus species have been identified, *African cassava mosaic virus*

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(ACMV), *East African cassava mosaic virus* (EACMV) and *Indian cassava mosaic virus* (ICMV) (Hong *et al.*, 1993; Swanson & Harrison, 1994). Recently, a fourth virus species was isolated in South Africa (Berrie *et al.*, 1998) with a proposed name of *South African cassava mosaic virus* (SACMV). CMD was first detected in Uganda in 1928 (Martin, 1928). Severe epidemics were reported between 1933 and 1944, although they were successfully controlled by the use of resistant cassava varieties and by sanitation of infected plants (Otim-Nape *et al.*, 1997). The situation remained stable until 1988, when an extremely severe epidemic of CMD developed, advancing from the north to the south of the country at a rate of approximately 20–25 km per year. This aggressive form of the disease has devastated cassava fields and caused food shortages and famine in a number of districts where the crop was the major staple (Otim-Nape *et al.*, 1996). A correlation exists between the presence of a new virus, recombinant between ACMV and EACMV, and the epidemic (Deng *et al.*, 1997; Zhou *et al.*, 1997). In order to have a better understanding of the dynamics of this epidemic, we performed a large sampling in Uganda in February 1997.

Based on observations of cassava grown in the field and in controlled growth-room conditions and results of PCR with oligonucleotide primers for genes encoded by both components A and B of cassava-infecting geminiviruses, we report here the molecular variability of cassava geminiviruses occurring in Uganda in 1997 and discuss the importance and role of recombination, pseudorecombination and synergism in the Ugandan CMD epidemic.

Methods

■ **Field sampling of infected cassava and whiteflies.** Cassava cuttings were collected from 80 different sites in Uganda during February 1997. Samples were collected from fields in the southern, central and northern regions of the country (Fig. 1). Cuttings 25–30 cm in length were taken from the central part of the hardwood stem of infected plants. At each field location, samples were collected from infected local cassava varieties and, whenever possible, cuttings displaying mild and severe symptoms were collected from the same variety. Whiteflies were collected on infected leaves and preserved in 80% ethanol in 1.5 ml Eppendorf tubes.

■ **Symptom reproduction in a controlled environment.** Cuttings collected from the field were planted in a growth chamber at 24 °C with a 14 h photoperiod and 70% relative humidity. They were watered twice a week and fertilized once a week with Peter's professional 15-16-17 nutrient solution. Disease symptoms were recorded daily on the first emerging leaves. The symptom severity on fully expanded leaves was scored by using the scale described by Fauquet & Fargette (1988).

■ **Virus inoculation on *Nicotiana benthamiana*.** In order to determine the severity of different virus isolates, 30-day-old *N. benthamiana* seedlings were inoculated with sap extracted from infected cassava leaves collected from Ugandan fields and sap of previously CMD-infected *N. benthamiana* leaves. The sap was diluted in ice-cold 0.1 M phosphate buffer, pH 7.0. One µg each of DNA-A (pCLV1.3A) and DNA-B (pCLV2B) of ACMV (Klinkenberg *et al.*, 1989), kindly

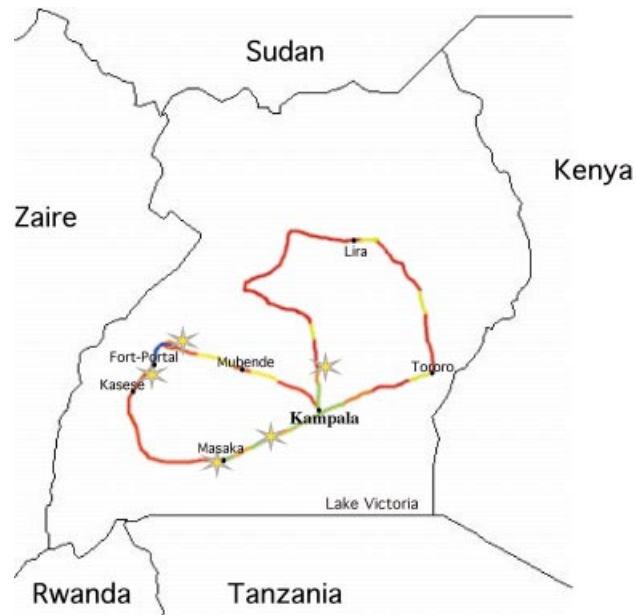


Fig. 1. Distribution of African cassava mosaic geminiviruses in Uganda in February 1997. Colours represent areas of occurrence of the different isolates identified in this study. EACMV-UG2/Svr DNA-A and EACMV-UG3 DNA-B (red) were widespread in the country whereas EACMV-UG2/Mid (yellow) was confined to isolated pockets and ACMV-UG was limited to certain areas: ACMV-UG/Svr (blue) in the highlands and ACMV-UG/Mid (green) along Lake Victoria. Mixed infections (orange) occurred at the contact zones between the main virus species, ACMV and EACMV. EACMV-UG1 DNA-A and -B and EACMV-UG3 DNA-A occurred at isolated sites (stars).

provided by John Stanley, John Innes Centre, UK, were also inoculated to *N. benthamiana* as a positive control. Inoculum was rubbed onto the surface of the first two fully expanded *N. benthamiana* leaves with carborundum. Symptom development was recorded daily and the third leaf from the apex was removed and frozen every 5 days for PCR and Southern blot analysis.

■ **Biolistic inoculation.** Clones of EACMV-UG2 DNA-A (pJSP-EA2) and EACMV-UG3 DNA-B (pJSP-EB3) were inoculated to 3-week-old cassava plantlets (cultivar TMS 60444) by using the Bio-Rad biolistic device. Gold particles were coated with 200 ng of each component as described by Garzón-Tiznado *et al.* (1993).

■ **DNA preparation.** Total DNA was extracted from whiteflies and from cassava and *N. benthamiana* leaves as described by Dellaporta *et al.* (1983).

■ **PCR.** In order to amplify the full-length coat protein (CP) gene, PCR with *Taq* DNA polymerase (Life Technologies/Gibco-BRL) was performed with a 94 °C denaturation step followed by 20 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C and an extension cycle of 10 min at 72 °C. A mixture of *Taq* DNA polymerase and cloned *Pfu* DNA polymerase (Stratagene) at a ratio of 15:1 was used to amplify the full-length viral DNA-A and -B with a first cycle of 4 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 58 °C and 3 min at 72 °C and a final extension cycle of 15 min. CMD-infected plants and whiteflies were screened by PCR amplification with the primers listed in Table 1.

■ **Plasmid construction.** Full-length copies of ACMV-UG DNA-A and -B, EACMV-UG2 DNA-A and EACMV-UG3 DNA-B were

Table 1. Primers used for PCR characterization and amplification of geminiviruses occurring in cassava plants and whitefly

Notation for degenerate oligonucleotides: R, A/G; Y, C/T; N, A/C/T/G; K, T/G; W, A/T. f.l.; Full-length. BV1 is the DNA-B virion-sense gene; BC1 is the DNA-B complementary-sense gene.

Primer	Sequence (5' → 3')	Target region
JSP001	ATGTCGAAGCGACCAGGAGAT	5' ACMV/EACMV CP
JSP002	TGTTTATTAATTGCCAATACT	3' ACMV CP
JSP003	CCTTTATTAATTTGTCCTACTGC	3' EACMV/SACMV CP
JSP004	ATCTCCTGGTCGCTTCGACAT	ACMV-UG DNA-A f.l.
JSP005	ATCATCATTCCACTCCAGGC	ACMV-UG DNA-A f.l.
JSP006	TGGTAGAATTCTTTGAAGTGTGAGGTA	EACMV-UG2 DNA-A f.l.
JSP007	GATTTGAATTCATGGGGGCCAAAGGGAC	EACMV-UG2 DNA-A f.l.
JSP008	GAATTCAGCCTCAGAGGTATACTG	ACMV-UG DNA-B f.l.
JSP009	GAATTCCTACTTAGAGGGCTACAC	ACMV-UG DNA-B f.l.
JSP010	AAGACGGATTCTTGTTAGAAGTGGAGAAA	EACMV-UG1/3 DNA-B f.l.
JSP011	CCACGGAATTCCTCCGACTGCTTCCTA	EACMV-UG1/3 DNA-B f.l.
JSP012	GTCCATATAGGTAARGTNATG	5' ICMV CP
JSP013	CCTGCTCCTTGCTNGCYTART	3' ICMV CP
JSP014	GCTGTCCCCATTGTCCARGGN	5' SACMV CP
JSP015	TGCCCAAAGTCTTGGGGGCGCATTG	EACMV-UG2 recombined frag.
JSP016	TGTCCAAAATCTTGAGGACTCGAA	EACMV-TZ recombined frag.
JSP017	TCCTCCTATTAATTGGAGACATTA	ACMV BC1
JSP018	CCTATATATAATACATAACAATT	ACMV BC1
MP 16	CCTCTAGATAATATTACYKRWKGRCC	Universal primer
MP 82	CGGAATTCYTGACCTTTCANGGNCCYTCR	Universal primer
Primers used for PCR discrimination of EACMV-UG1 and EACMV-UG2 DNA-B		
JSP019	TAGACTTCCATTCAAAATTTGAAT	EACMV-UG1 CR
JSP020	CTCAATGAATGTGAAAGGCCAAACG	EACMV-UG3 CR-B
JSP021	TGCCTTTCACATTCATTGAGGN	EACMV-UG3 CR-B
JSP022	CCTATTTACACATATGCCATTGGG	EACMV-UG1 CR
JSP023	TACATCGGCCTTTGAGTCGCATGG	EACMV-UG1/3 BC1
JSP024	CAGACACCCTCAAGCTTAATA	EACMV-UG1/3 BV1

amplified by PCR and cloned into pBluescript II KS (+). Unique *Bam*HI and *Hind*III sites were used respectively for cloning ACMV-UG DNA-A and EACMV-UG2 DNA-A and *Eco*RI for ACMV-UG DNA-B and EACMV-UG3 DNA-B. Head-to-tail partial repeats of these clones were constructed as described by Von Arnim & Stanley (1992).

■ **Southern blot analysis.** Total DNA (4 µg) was electrophoresed on a 1% agarose gel without ethidium bromide and transferred to nylon membranes. Viral DNA was detected by using specific radiolabelled probes of ACMV-UG DNA-A and -B, EACMV-UG2 DNA-A and EACMV-UG3 DNA-B.

■ **Sequence determination and analysis.** The complete DNA sequences of ACMV-UG DNA-A and -B, EACMV-UG2 DNA-A and EACMV-UG3 DNA-B from mild and severely CMD-affected cassava plants were sequenced by the dideoxynucleotide chain-termination method on an ABI automatic sequencer. In addition, part of the EACMV-UG3 DNA-A, cloned using the TA cloning kit (Invitrogen), and the full-length EACMV-UG1 DNA-B were also sequenced as described above. Sequences obtained were compared by using the cluster option of the multiple sequence alignment (MegAlign) program within the DNASTAR package for Apple Macintosh computers. An identical consensus tree was obtained when phylogenetic analysis was performed by a cladistic parsimony method using the computer program PAUP 3.1.1 (Swofford,

1993). Ten thousand bootstrap replications were performed to place confidence estimates on the groups contained in the tree.

Results

Sample collection

The route followed to collect cuttings of CMD-infected cassava plants in Uganda is shown in Fig. 1. Samples representing the south-western, central and part of the northern regions were obtained. The extreme north of the country could not be prospected as it was inaccessible at the time of collection in February 1997.

More than 90% of the cassava plants showed severe CMD symptoms in almost all of the fields in all regions visited. However, mild symptoms could also be observed in some plants scattered throughout some fields. The northern border of Lake Victoria represented an exception, as generally only mild CMD symptoms were found in that area. A total of nine cultivars were collected, with Kameza in the south-western and Ebwanatereka in the south-eastern region being the most

strongly represented (38 and 27% of the collection, respectively).

Establishing cassava cuttings in growth chambers

Symptoms of infected cassava samples collected in the field were reproduced in controlled conditions to assess symptom variability linked to the environment. From a total of 183 cuttings collected, 129 (70.5%) were successfully established in the growth chamber. Of the 54 cuttings that failed to grow, 55% were from plants severely affected by CMD in the field and 45% were from plants that displayed mild symptoms. Therefore, failure of the cuttings to establish in the growth chamber was not related to disease severity. Some cuttings showed symptom variations in the growth chamber compared with the field-assessed symptoms. Consequently, only plants showing clearly reproducible symptoms on two different cuttings after 70 days in the growth chamber were selected for subsequent analysis.

CMD symptoms on cassava plants

Fig. 2 shows the different types of disease symptoms observed on cassava plants growing in the field and in the growth chamber. In both cases, cassava plants expressing mild symptoms developed normally with leaves showing mild, light-green mosaic symptoms. In contrast, plants expressing severe symptoms displayed extreme shrinking of the leaves, along with distortion at the bases of the leaflets and distinct chlorosis. In very severe cases, the leaves became desiccated and the young plants died. The proportion of disease-related death of cassava plants was estimated periodically for the predominant cultivars in the collection. Cultivar Ebwanatereka was very susceptible to CMD, as 32% of cuttings of that cultivar died 90 days after establishment in the growth chamber. Other cultivars were less susceptible, with approximately 15% of the Ssenyonjo and Kameza cuttings dying within 90 days, while only 8% of Machunde cuttings had died during the same period.

Molecular characterization of cassava geminiviruses in Uganda

Based on the published nucleotide sequences of ACMV-KE (Stanley & Gay, 1983), EACMV-TZ and EACMV-UG2 DNA-A (Deng *et al.*, 1997; Zhou *et al.*, 1997), specific and degenerate primers (Table 1) were employed to characterize the Ugandan cassava collection. Using discriminating primers to amplify the CP gene, 48% of the cassava samples were found to be infected by EACMV-UG2 alone, 28% by ACMV-UG alone and 7% with both ACMV-UG and EACMV-UG2 and 8% were triple-infected with ACMV-UG, EACMV-UG1 (a non-recombined sequence similar to EACMV from Tanzania) and EACMV-UG2. For 9% of the samples, no signal was detected for any of

the primers pairs employed. None of the cassava samples tested in this study was infected by EACMV-UG1 alone.

Cassava samples that failed to produce a signal with the specific primers (JSP001, 002, 003) showed mild symptoms in the field and no symptoms in the greenhouse. These samples were then tested with the degenerate primers MP16 and MP82 (Padidam *et al.*, 1995). A fragment of approximately 500 bp was amplified, stretching from the stem-loop of the CR to the 5' end of the core region of the CP. This suggested the presence of an as-yet undescribed geminivirus, which we shall call provisionally EACMV-UG3. Only a partial DNA-A sequence of this virus was obtained in this study, stretching from 150 nt upstream of the nonanucleotide to the 5' end of the core region of the CP.

None of the CMD-affected plants from our collection yielded PCR products with CP primers specific for ICMV or SACMV (Table 1), suggesting that neither ICMV nor SACMV is present in Uganda.

The relationship between Ugandan cassava geminiviruses, CMD symptom severity and virus DNA accumulation

As described previously, cassava plants displaying mild symptoms could be found in fields where the severe condition was also prominent. In order to understand this situation better, a relationship was established between the virus type, ACMV or EACMV, found in the plants by PCR analysis and symptom severity (Fig. 2F). Eighty per cent of ACMV-UG-positive plants exhibited very mild symptoms and 20% showed intermediate symptoms. Sixty-seven per cent and 33% of EACMV-UG2-infected plants were respectively severely and moderately affected. Noticeably, all samples found to be infected by both ACMV-UG and EACMV-UG2 were very severely affected (10% scored 3 and 90% scored 4), suggesting a synergistic interaction between these two virus species. The suffix -UG/Mld or -UG/Svr was used to discriminate between the putative viruses present in the different inocula.

Cassava plants of the cultivar Ebwanatereka displaying mild and severe symptoms were selected for further study. Crude sap was extracted from infected leaves and tested for its ability to transmit the viruses to *N. benthamiana*. In the case of plants with mild symptoms, only areas of the leaves showing symptoms were used for crude sap preparation. Using diluted crude sap solutions (1 g leaf tissue ground in 10 ml buffer), infection of *N. benthamiana* could be obtained with both ACMV-UG/Svr and EACMV-UG2/Svr. A more concentrated sap solution of at least 5 g/10 ml was necessary to obtain symptoms with ACMV-UG/Mld. For sap extracted from cassava infected by EACMV-UG2/Mld, symptoms were barely detectable in *N. benthamiana*, regardless of the inoculum concentration employed. However, specific PCR products attesting to the presence of the respective viruses could be

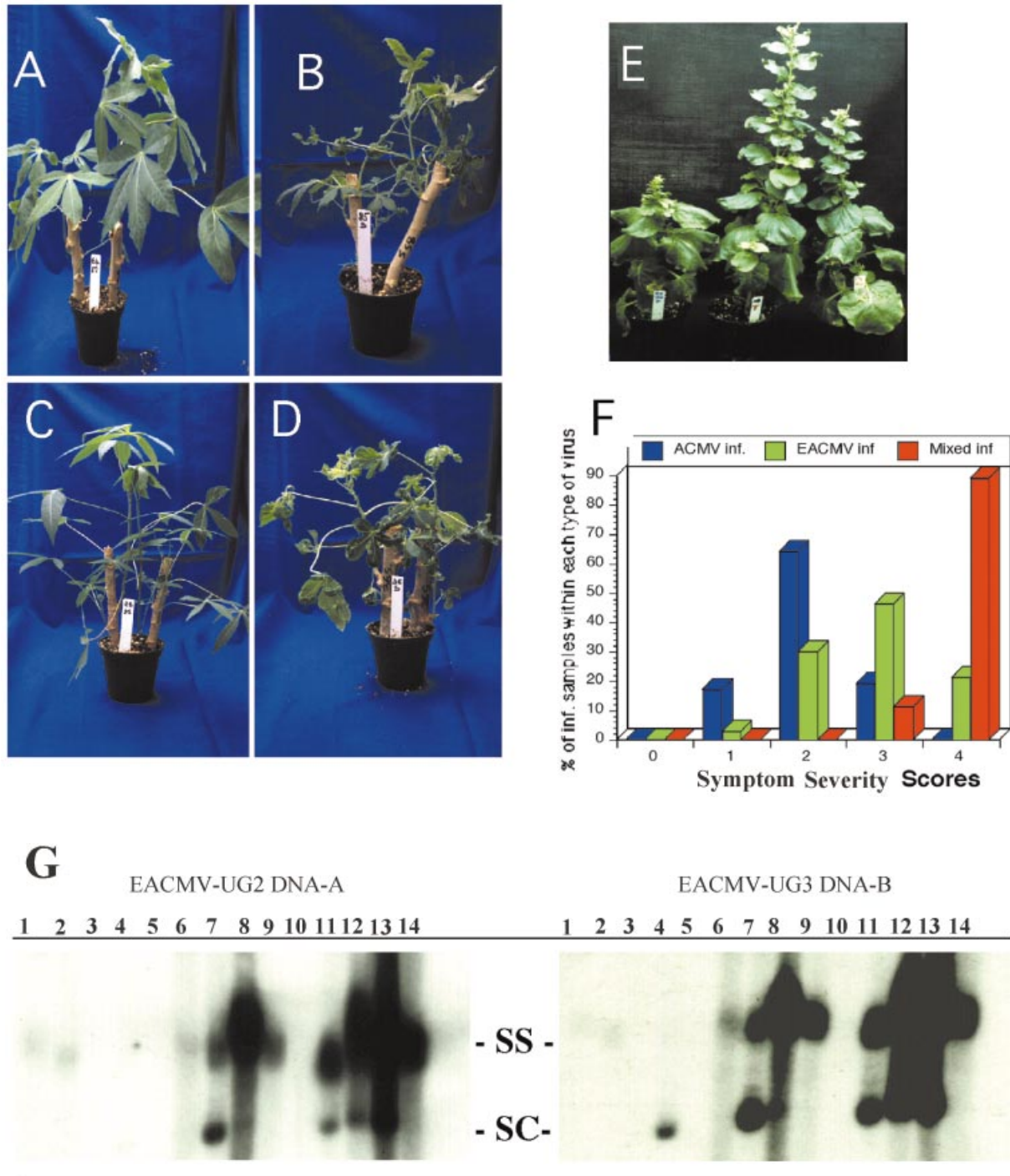


Fig. 2. Relationship between Ugandan cassava geminiviruses, symptom severity and viral DNA accumulation. (A)–(D) Plants grown from cassava cuttings infected with ACMV-UG/Mld (A), ACMV-UG/Svr (B), EACMV-UG2/Mld (C) or EACMV-UG2/Svr (D). (E) *N. benthamiana* plants inoculated with EACMV-UG2/Svr (left), ACMV-UG/Svr (right) and a control (centre). (F) Distribution of symptom severity on cassava samples collected in Uganda in February 1997, according to the infected virus (based on PCR characterization with the CP-specific primers JSP001, JSP002 and JSP003). (G) Southern blot analysis showing relationships between symptom severity and virus accumulation in naturally infected cassava plants. The left and right panels show the level of accumulation of EACMV-UG2 DNA-A and EACMV-UG3 DNA-B in plants infected by EACMV-UG2/Mld (lanes 1–6), EACMV-UG2/Svr (7, 9, 11, 14) or doubly infected by ACMV-UG and EACMV-UG2 (8, 12, 13) and the non-infected control (10). SS, Single-stranded; SC, supercoiled.

obtained on systemically affected leaves of all inoculated plants at 10 days post-inoculation for ACMV-UG and approximately 30 days post-inoculation for EACMV-UG2.

ACMV-UG and EACMV-UG2 induced different types of symptoms in *N. benthamiana* (Fig. 2E). ACMV-UG-infected plants did not show the typical mosaic and severe leaf-curling symptoms of ACMV-KE (Klinkenberg *et al.*, 1989) characteristic in *N. benthamiana* plants, but caused moderate leaf curling at the base of systemically infected leaves and/or chlorotic spots on inoculated leaves. EACMV-UG2-infected plants were reduced in height compared with ACMV-UG-infected plants or control, non-infected plants (Fig. 2E). Systemic leaves in EACMV-UG2/Svr-infected plants showed an approximately 40% reduction in size compared with similar leaves of wild-type *N. benthamiana* and chlorotic patches rather than the mosaic typical of ACMV were present on older leaves.

The relationship between virus accumulation and symptom severity was investigated by using Southern blot analysis. Cassava samples infected by EACMV-UG2/Mld, EACMV-UG2/Svr and mixed-infected by ACMV-UG and EACMV-UG2 were used. Four µg total DNA extracted from infected leaves of cultivar Ebwanatereka was used for Southern hybridization. A positive correlation between symptom severity and virus accumulation was obvious (Fig. 2 G). EACMV-UG2/Mld was barely detectable compared with EACMV-UG2/Svr, whereas samples with mixed infections gave stronger signals, indicating probable synergistic interaction between ACMV-UG and EACMV-UG2.

Sequence analysis and comparison with selected viruses

Coat protein (CP). The CP gene sequences of the four strains identified in our study were compared to published sequences. Results indicated that the CP nucleotide sequences from ACMV-UG/Mld, ACMV-UG/Svr, EACMV-UG2/Mld and EACMV-UG2/Svr were nearly identical within each virus species. In order to test whether the CP gene sequences of all EACMV-UG2 isolates found in our collection were similar to the published sequence of the recombinant EACMV-UG (Deng *et al.*, 1997; Zhou *et al.*, 1997), primers specific to the recombinant fragment (JSP015 and JSP016) were designed to amplify selectively the CP of EACMV-UG1-like or EACMV-UG2-like molecules. All EACMV-UG2 isolates, irrespective of symptom severity, were found to contain the same ACMV recombinant fragment in their CP. Of the 129 samples tested, EACMV-UG1-like CP sequences were amplified from only five samples that were also infected by ACMV-UG, EACMV-UG2 and EACMV-UG3.

Complete DNA-A sequences. Complete nucleotide sequences of the DNA-A molecules of the different Ugandan ACMV and EACMV strains were compared with published sequences

Table 2. Nucleotide sequence identity (%) of complete DNA-A sequences of selected cassava geminiviruses

Values over 80% are shown in bold.

Virus isolate	1	2	3	4	5	6	7	8	9
1. ACMV-UG/Mld	–	97	97	96	69	68	64	64	66
2. ACMV-UG/Svr		–	97	97	69	69	64	64	66
3. ACMV-KE			–	96	68	68	63	64	66
4. ACMV-NG				–	69	68	64	64	66
5. EACMV-UG2/Mld					–	99	91	90	85
6. EACMV-UG2/Svr						–	92	91	85
7. EACMV-KE							–	94	86
8. EACMV-TZ								–	85
9. EACMV-MW									–

Table 3. Nucleotide and amino acid sequence identities (%) of BC1 and BV1 of selected cassava geminiviruses

Numbers in parentheses correspond to percentage amino acid sequence identities. The part of the matrix above the diagonal shows BC1 comparisons and the part below the diagonal shows BV1 comparisons. Values over 80% are in bold.

Virus isolate	1	2	3	4	5
1. ACMV-KE	–	97 (96)	96 (97)	46 (53)	46 (53)
2. ACMV-NG	95 (92)	–	97 (98)	45 (54)	46 (53)
3. ACMV-UG	96 (96)	96 (94)	–	45 (53)	46 (53)
4. EACMV-UG1	37 (34)	35 (34)	35 (34)	–	98 (97)
5. EACMV-UG3	37 (35)	36 (36)	36 (35)	99 (98)	–

(Table 2). Comparison of these sequences revealed a higher percentage identity within each virus species ($\geq 85\%$) than between them (63–69%). The sequences were as expected and no other recombination was found in ACMV-UG or EACMV-UG2 DNA-A.

Evidence that the Ugandan EACMV-UG3 DNA-B has arisen by a recombination event. The complete nucleotide sequences of the DNA-B components of EACMV-UG1 and EACMV-UG3 were determined. The nucleotide sequences and the deduced amino acid sequences of the BC1 and BV1 genes of both DNA-B were compared with the B components of ACMV isolates from Kenya (Stanley & Gay, 1983) and Nigeria (Morris *et al.*, 1990) (Table 3). The results confirmed that ACMV and EACMV are two different species (identity $< 75\%$) and showed that the BV1 gene is more variable between the two virus species, with $\leq 35\%$ identity compared with $\leq 53\%$ for the BC1 gene at the amino acid level. EACMV-UG1 and EACMV-UG3 DNA-B were 96% identical, with the major differences localized in the CR, within the 120 nt upstream of the nonnucleotide.

Table 4. Nucleotide sequence identities (%) between the CR of selected cassava geminiviruses

CR-A indicates the common region of the A component and CR-B indicates the common region of the B component. Values over 80% are in bold.

Virus	ACMV-UG CR-B	ACMV-KE CR-B	ACMV-NG CR-B	EACMV-UG1 CR-B	EACMV-UG3 CR-B
ACMV-UG CR-A	90	90	91	45	47
ACMV-KE CR-A	89	91	90	45	47
ACMV-NG CR-A	90	90	94	43	43
EACMV-KE CR-A	41	44	43	87	60
EACMV-TZ CR-A	42	43	44	94	65
EACMV-MW CR-A	43	47	45	82	54
EACMV-UG2 CR-A	42	45	45	91	60
EACMV-UG3 CR-A	46	47	47	65	93
EACMV-UG1 CR-B	41	44	44	–	70
EACMV-UG3 CR-B	44	45	44	70	–

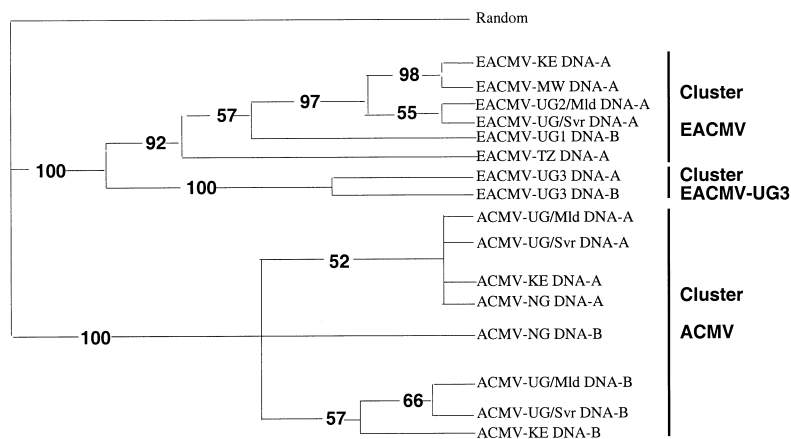


Fig. 3. Consensus phylogenetic tree (10 000 bootstrap replications) obtained from comparison of the CR sequences of selected cassava geminiviruses.

The CR contains various features characteristic of begomoviruses and presents a high sequence similarity ($\geq 90\%$) between the A and B components. Table 4 presents a comparison of the nucleotide sequences in the CR of the viruses identified in this study (including EACMV-UG3 CR-A) with the corresponding CR from previously published sequences. Fig. 3 presents the phylogenetic tree obtained from the comparison. The results show three important clusters. Cluster EACMV groups all EACMV CR-A sequences (excluding EACMV-UG3 CR-A) with EACMV-UG1 CR-B. EACMV-UG3 CR-B and EACMV-UG3 CR-A form the EACMV-UG3 cluster, with 93% sequence identity. The ACMV cluster groups all the ACMV CR-A and CR-B sequences with a sequence identity above 90%.

EACMV-UG2 CR-A and EACMV-UG3 CR-B show only 60% sequence identity compared with EACMV-UG2 CR-A and EACMV-UG1 CR-B (91%) or EACMV-UG3 CR-B and EACMV-UG3 CR-A (93%). These results provide evidence that EACMV-UG3 CR-B could have resulted from recombination with another species.

Natural pseudorecombination between Ugandan cassava geminiviruses

In field-collected cassava plants. Although earlier results showed a very low sequence identity in the CR of EACMV-UG2 DNA-A and EACMV-UG3 DNA-B, we found that, when primers specific for ACMV DNA-B, JSP017 and JSP018, were used to characterize the collection, all samples containing either ACMV DNA-A alone or ACMV DNA-A in mixed infection with EACMV-UG2 DNA-A were found also to contain ACMV DNA-B. Using four different sets of primers (Table 1) respectively specific for EACMV-UG3 DNA-B (JSP020 and 024; JSP021 and 023) or EACMV-UG1 DNA-B (JSP019 and 024; JSP022 and 023), all samples infected either with EACMV-UG2 DNA-A alone or with EACMV-UG2 DNA-A associated with ACMV-UG DNA-A were always found to contain EACMV-UG3 DNA-B instead of EACMV-UG1 DNA-B. EACMV-UG1 DNA-B was detected in only five samples in mixed infection with ACMV-UG DNA-A and -B, EACMV-UG1 DNA-A, EACMV-UG2 DNA-A and EACMV-

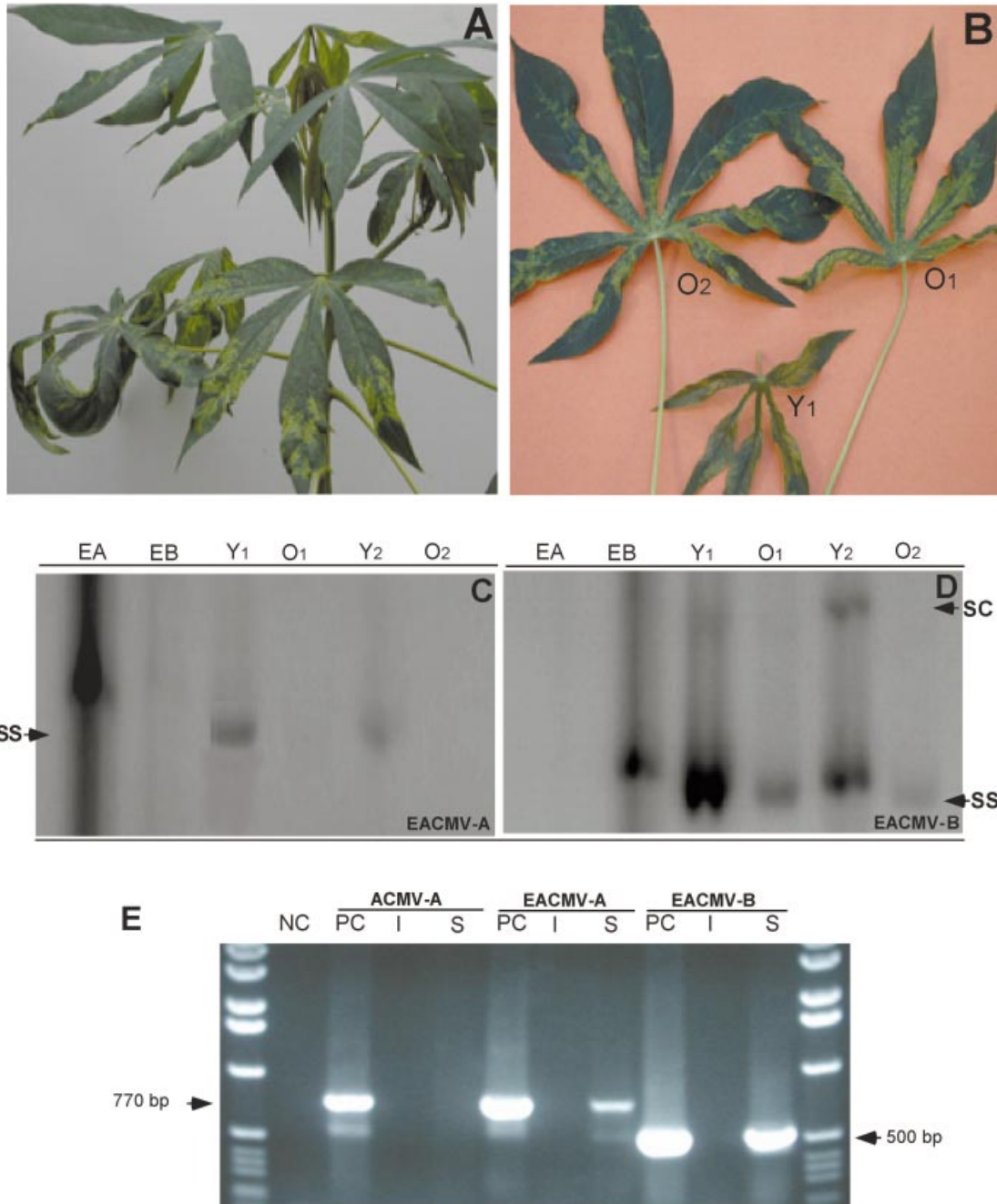


Fig. 4. Infectivity of the pseudorecombinant EACMV-UG2 DNA-A + EACMV-UG3 DNA-B to cassava by biolistic inoculation. (A) Branch showing CMD symptoms on systemically infected leaves. (B) CMD symptoms on young (Y_1) and old (O_1 , O_2) systemically infected leaves. (C)–(D) Southern blot analysis showing virus accumulation in young and old systemically infected leaves. EA and EB correspond to the positive controls. Y_1 , O_1 , Y_2 and O_2 correspond to young (Y) and old (O) systemically infected leaves of two plants (1 and 2). SS, Single-stranded; SC, supercoiled. (E) PCR detection of EACMV-UG2 DNA-A and EACMV-UG3 DNA-B in systemically infected leaves. NC and PC, Negative and positive controls; I and S, inoculated and systemically infected leaves of the same cassava plant.

UG3 DNA-A and -B. Some cassava plants that were EACMV-UG2 CP-positive and ACMV CP-negative were also detected as positive for ACMV DNA-B by PCR.

In field-collected whiteflies. PCR amplification of DNA from

individual whiteflies using the same primers employed to screen the cassava cuttings confirmed the presence of ACMV-UG and EACMV-UG2 as well as the absence of ICMV and SACMV in Uganda. Of 49 whiteflies assessed, assortments of ACMV DNA-A and -B or EACMV-UG2 DNA-A and

EACMV-UG3 DNA-B were found, as well as several other combinations of components A and B, indicating that the transmission of cassava geminiviruses by their whitefly vector is not specific.

In biologically inoculated cassava plants. As described above, EACMV-UG2 CR-A and EACMV-UG3 CR-B were only 60% identical. However, the EACMV-UG2 A component and EACMV-UG3 B component were always found together in cassava plants collected from the field. This suggested that, despite the low identity of their CR, EACMV-UG2 DNA-A could replicate EACMV-UG3 DNA-B. We investigated the infectivity of the pseudorecombinant EACMV-UG2 DNA-A + EACMV-UG3 DNA-B in cassava plants with clones of the respective virus components (Fig. 4). We found that this pseudorecombinant virus is infectious and that both components replicate in inoculated cassava plants (Fig. 4 C–E), thereby confirming the existence of natural pseudorecombination between these cassava geminiviruses. A PCR was performed to demonstrate that, despite its low accumulation (Fig. 4C), EACMV-UG2 DNA-A is present in the systemically infected leaves, as confirmed by amplification with primers JSP001 and JSP003 (Fig. 4E).

Geographical distribution of cassava geminiviruses in Uganda

Fig. 1 shows the geographical distribution of the cassava geminiviruses identified by PCR in Uganda. All plant samples collected from the central and north-eastern regions of Uganda were found to contain EACMV-UG2 DNA-A and EACMV-UG3 DNA-B. Of these, 24% also contained ACMV-UG DNA-A and -B, but none were infected by ACMV-UG DNA-A and -B alone. CMD symptoms in these regions were mostly severe, but plants with mild symptoms (infected by EACMV-UG2 DNA-A and EACMV-UG3 DNA-B) were also observed.

Five zones were identified in the southern part of the country. The first zone, ranging from the northern region of Kampala to the western vicinity of Masaka, along the border of Lake Victoria, was characterized by the occurrence of ACMV-UG DNA-A and -B and was associated with mild symptoms on cassava plants. The few plants displaying severe symptoms in this region were found to contain a combination of ACMV-UG DNA-A and -B, EACMV-UG1 DNA-A and -B, EACMV-UG2 DNA-A, EACMV-UG3 DNA-B and EACMV-UG3 DNA-A and occurred in isolated, scattered sites. The second zone spanned from west Masaka to 46 km north of Kasese. Within this region, all cassava samples analysed by PCR were infected only by EACMV-UG2 DNA-A and EACMV-UG3 DNA-B and displayed mostly severe symptoms. The third zone covered a small area only 17 km long between the towns of Bunyangabu and Fort Portal and was characterized by the presence of ACMV-UG DNA-A and -B and was associated only with severe CMD symptoms. A fourth zone of 26 km beyond Fort Portal was characterized by plants showing

severe CMD symptoms, which were found to contain either ACMV-UG DNA-A and -B, EACMV-UG2 DNA-A and EACMV-UG3 DNA-B or both viruses combined with EACMV-UG1 DNA-A and -B and EACMV-UG3 DNA-A. Finally, a fifth zone, ranging from the fourth zone back to Kampala, was characterized by the presence of EACMV-UG2 DNA-A and EACMV-UG3 DNA-B and was associated with variable CMD symptoms.

Discussion

The present study of cassava cultivars sampled in Uganda in February 1997 confirmed the presence of ACMV-UG, EACMV-UG1 (non-recombinant) and EACMV-UG2 (recombinant in the CP) and revealed the occurrence of another virus, which we have called EACMV-UG3 in this report. The southwestern region, which was infected predominantly by ACMV-UG in 1995, had been invaded by EACMV-UG2 in February 1997.

Symptoms observed on cassava plants growing in Ugandan fields could be reproduced on cassava plants growing in a controlled environment and on mechanically inoculated *N. benthamiana* plants. There was a strong positive correlation between symptom severity and viral DNA accumulation, suggesting the existence of mild and severe strains for both virus species ACMV and EACMV in Uganda, with a defined geographical distribution of the infection types at the time of the survey: ACMV-UG/Svr was restricted to the highlands, ACMV-UG/Mld to the central region along the shore of Lake Victoria, EACMV-UG2/Mld was confined to isolated pockets in different sites while EACMV-UG2/Svr had invaded all of the areas of Uganda sampled where the CMD epidemic occurred. The geographical coverage of the virus species reflected the extent to which the epidemic had covered the different parts of the country. The dominance of ACMV-UG/Mld in areas around Lake Victoria was due to the fact that the epidemic had not yet reached these areas at the time of the survey. Recent observations (G. W. Otim-Nape, unpublished) indicate that this area has now been reached by the epidemic and the different virus species are now found there.

PCR and sequence analysis confirmed that mild and severe EACMV-UG2 strains contained the same recombinant ACMV fragment in their CP, irrespective of the CMD symptoms they induced in cassava plants. The interspecies exchange event in the CP gene is therefore not related directly to symptom expression, unless subsequent independent mutations in the severe strain have abolished the symptom severity that was produced initially by this recombination.

The contact zones between the two main geminivirus species found in Uganda revealed several interesting features. In particular, it is obvious that mixed infection occurs frequently in cassava plants in nature and provides the opportunity for recombination to occur between viruses. EACMV-UG1, which is very similar in sequence to EACMV-

TZ within its CP gene, was always found mixed with ACMV-UG, EACMV-UG2 and EACMV-UG3, and therefore could be one of the putative parents of the recombinant EACMV-UG2. This was not obvious from previous reports, where sampling did not allow the detection of EACMV-UG1 in Uganda (Harrison *et al.*, 1997).

Forty-five per cent of EACMV-UG2-infected cassava plants presented very severe CMD symptoms. These samples were PCR-positive for the presence of EACMV-UG2 DNA-A, EACMV-UG3 DNA-B and ACMV-UG DNA-B, but negative for ACMV-UG DNA-A. This suggests a natural pseudorecombination between EACMV-UG2 DNA-A and ACMV-UG DNA-B, in a manner already demonstrated for several bipartite geminiviruses (Hill *et al.*, 1998; Hou *et al.*, 1998). Pseudorecombination was confirmed in biologically inoculated cassava plants using infectious clones, proving that EACMV-UG2 DNA-A can replicate EACMV-UG3 DNA-B despite the low identity of their CR.

The intensity of CMD symptoms could be correlated with the synergistic interaction observed between ACMV-UG and EACMV-UG2. All samples doubly infected with ACMV DNA-A and -B and EACMV DNA-A and -B within our Ugandan cassava collection produced extremely severe symptoms compared with plants infected with only one virus species. To date, we have not tried to reproduce this synergistic interaction with infectious clones. However, Southern blot analysis of naturally CMD-infected cassava plants showed a greater accumulation of EACMV-UG2 DNA-A and EACMV-UG3 DNA-B in mixed-infected plants compared with singly infected plants. Such a synergistic interaction between ACMV and EACMV has been suggested by Harrison *et al.* (1997) and demonstrated by Fondong *et al.* (2000) with infectious clones of ACMV-CM and EACMV-CM isolated from Cameroon.

Another important result described in this paper is the evidence for interspecies recombination provided by EACMV-UG3 DNA-B. This DNA species is highly similar to the EACMV-UG1 DNA-B component with the exception of the CR, which contains considerable differences that may have resulted from recombination with another geminivirus species. Despite the low nucleotide sequence identity in their CR (60%), we have shown that the pseudorecombinant virus EACMV-UG2 DNA-A + EACMV-UG3 DNA-B is infectious to biologically inoculated cassava plants and is the combination found most frequently in nature, probably due to its tendency to accumulate more in the youngest leaves (Fig. 4C) that are targeted preferentially by the whitefly vector. The close association between EACMV-UG2 DNA-A and EACMV-UG3 DNA-B and the fact that these two molecules are the most widespread in Uganda provides evidence of their essential role in the severe CMD epidemic in Uganda. It appears that the association of EACMV-UG2 DNA-A and EACMV-UG3 DNA-B is privileged over the homologous combination and this could be a key to the CMD epidemic, as the pseudorecombinant might replicate more, accumulate more

DNA and consequently cause more severe symptoms. This hypothesis could be supported by the fact that we observed a higher level of EACMV-UG3 DNA-B accumulation in cassava leaves compared with EACMV-UG2 DNA-A (Fig. 4C). This observation is consistent with the severe symptoms that develop in the young systemic leaves of infected plants. We do not currently understand the significance of the dominance of EACMV-UG3 DNA-B accumulation over EACMV-UG2 DNA-A, but this will be investigated in future work.

Specific or preferential transmission of the recombinant (CP) strain compared with EACMV-UG1 could explain the invasion of Uganda by EACMV-UG2 and the disappearance of the non-recombinant EACMV-UG1. Spread of EACMV-UG2 within areas where cassava plants were already infected by ACMV would then have been enhanced through its synergistic interaction with ACMV, leading to development of the CMD epidemic.

In summary, a combination and/or the sequential occurrence of several phenomena could explain the Ugandan CMD epidemic. (i) Synergism between members of the two species ACMV and EACMV is certainly a major possible factor, as it increases DNA accumulation of both viruses and therefore their capacity to be transmitted by whiteflies. (ii) The fact that the most prevalent virus in the epidemic (EACMV-UG2) is a recombinant for CP with ACMV-UG is certainly not a coincidence. However, the reason for its success is not evident, in view of the fact that we found a very mild strain of EACMV-UG2 in Uganda that possessed the same recombination. The recombinant CP could have the same level of replication as the non-recombinant but might possess similar whitefly transmission-properties to those of ACMV. This, however, remains to be proven. Subsequent mutations and selection by farmers of plants that displayed mild symptoms could easily explain the occurrence of mild EACMV-UG2 isolates. (iii) For the first time, we demonstrate that it is possible in nature to find stable pseudorecombinants between the A and B components of strains of the same species (EACMV-UG2 DNA-A and EACMV-UG3 DNA-B) or between A and B components of different species (ACMV and EACMV). This system offers an additional possibility of survival for the viruses and another source of virus biodiversity.

These biological findings show that more than one event is probably necessary to start such CMD epidemics. However, more experimentation will be necessary to understand better the dynamics of this complex biological situation. Infectious clones of ACMV and EACMV that are now available will be used for this purpose.

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