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Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon

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Stem cuttings were collected in Cameroon from cassava plants displaying cassava mosaic disease (CMD) symptoms. The nature of the viruses present was determined by using the PCR with primers specific for the coat protein (CP) genes of African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV). All samples were infected by ACMV and eight of the 50 samples were infected by both ACMV and an EACMV-like virus. The complete nucleotide sequences of DNA-A and -B of representative ACMV and EACMV-like viruses were determined. The DNA-A component of the EACMV-like virus contained evidence of recombination in the AC2–AC3 region and DNA-B also contained evidence of recombination in BC1. However, both components retained gene arrangements typical of bipartite begomoviruses. When *Nicotiana benthamiana* plants were doubly inoculated with these Cameroon isolates of ACMV and EACMV (ACMV/CM, EACMV/CM) by using sap from cassava plants or infectious clones, the symptoms were more severe than for plants inoculated with either virus alone. Southern blot analysis of viral DNAs from infected plants showed that there were significantly higher levels of accumulation of both ACMV/CM components and, to a lesser extent, of EACMV/CM components in mixed-infected plants than in singly infected plants. These results strongly suggest the occurrence of a synergistic interaction between the two viruses.

Introduction

Cassava mosaic disease (CMD) occurs in all cassava (*Manihot esculenta* Crantz)-producing regions of Africa, India and Sri Lanka, resulting in annual yield losses estimated at 1 billion pounds sterling (Fargette *et al.*, 1988). CMD is caused by viruses belonging to the genus *Begomovirus* of the family *Geminiviridae*, which are characterized by small, geminate particles containing circular, single-stranded DNA molecules (Briddon & Markham, 1995). The viruses are transmitted by the whitefly *Bemisia tabaci* and spread through infected cuttings, which are the usual mode of cassava propagation. Swanson & Harrison (1994) identified three groups of cassava mosaic viruses on the basis of their reaction to monoclonal antibodies. Group A is limited to West Africa, Burundi, Chad, Uganda and the western part of Kenya, whereas group B occurs in Malawi, Madagascar, Zimbabwe and the eastern parts of Kenya and Tanzania. Group C is restricted to India and Sri Lanka. Because of considerable differences in their nucleotide sequences, these groups of viruses are now identified as different virus species (Hong *et al.*, 1993) and are named African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV) and Indian cassava mosaic virus (ICMV), respectively.

The genomes of ACMV and ICMV are composed of two similar-sized DNAs (DNA-A and DNA-B). The complete nucleotide sequences of several isolates of these virus species have been determined [ACMV from Kenya (ACMV/KE) and Nigeria (ACMV/NG) and ICMV] (Stanley & Gray, 1983; Morris *et al.*, 1990; Hong *et al.*, 1993). Only DNA-A of EACMV has been cloned [from isolates from Tanzania

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(EACMV/TZ), Uganda (EACMV/UG), Kenya (EACMV/KE) and Malawi (EACMV/MW)] (Deng *et al*., 1997; Zhou *et al*., 1997, 1998*b*).

Zhou *et al*. (1997) and Deng *et al*. (1997) reported a new cassava mosaic virus in Uganda (EACMV/UG1), which is considered to be a natural recombinant between ACMV and EACMV. This virus is associated with the severe Ugandan epidemic of CMD, which is characterized by a severe effect on plants and high populations of whiteflies and is reported to be advancing into hitherto-unaffected areas to the south. CMD in South Africa is caused by yet another distinct virus species, South African cassava mosaic virus (SACMV; Berrie *et al*., 1998).

Recently, Fondong *et al*. (1998) reported the occurrence of unusually severe symptoms of CMD on cassava in Cameroon that are associated with mixed infections of ACMV and EACMV-like species. We report here the complete sequence of components A and B from two viruses associated with CMD in Cameroon. DNA-A of the EACMV-like virus species was found to contain a recombinant fragment originating from an unknown virus species. Similarly, the DNA-B component of the EACMV-like virus species also showed evidence of recombination with a DNA-B component of an EACMV/UG-like virus similar to the one isolated in Uganda (unpublished data).

In nature, mixed virus infections occur in the same plant, with biological and epidemiological implications. Studies have shown that a synergistic interaction between potato virus X (PVX) and potato virus Y (PVY) resulted in enhanced PVX replication and severe necrosis in *Nicotiana tabacum* leaves (Damirdagh & Ross, 1967). Synergism has also been reported between potyviruses and maize chlorotic mottle virus (*Tombusviridae*; *Machlomovirus*) (Goldberg & Brakke, 1987), as well as between the comoviruses cowpea mosaic virus and bean pod mottle virus (Anjos *et al*., 1992). Recently, Harrison *et al*. (1997) reported the occurrence of ACMV and EACMV in plants with very severe symptoms in Uganda, Tanzania and southern Sudan and suggested the possibility of synergism between the two viruses. We report here, for the first time, molecular evidence for such synergism between two geminiviruses (ACMV/CM and EACMV/CM), in which cassava plants co-infected by the two viruses develop more severe symptoms in the field and in the growth chamber compared with plants infected by either virus alone.

Methods

■ **Sample collection.** Hardwood cassava stem cuttings, 20 cm in length, were collected from infected plants in three cassava-growing regions of Cameroon. Thirty-five samples were collected in the south-western rainforest region around Kumba, Ekona and Buea, 13 in Dschang in the western highlands and two in the savannah region near Ngaoundere. Cuttings were collected from plants displaying distinct disease symptoms. The cuttings were planted in the growth chamber and symptoms were observed on newly developed leaves.

■ **PCR.** Total DNA was extracted from young cassava leaves that showed CMD symptoms as described by Dellaporta *et al*. (1983). Oligonucleotide primers used to amplify the viral DNA are listed in Table 1. PCR was performed with the GIBCO BRL kit as recommended by the manufacturer. The PCR conditions for coat protein (CP) gene amplification were 2 min at 94 °C and then 30 cycles of 1 min at 94 °C, 1 min at 45 °C and 1 min at 72 °C followed by a final extension period of 10 min at 72 °C. A 700 bp fragment of the 5′ end and the intergenic region of EACMV/CM BC1 was amplified by using degenerate primers (Table 1) designed from published sequences of B components of begomoviruses. The PCR conditions for amplifying this fragment were similar to those used for amplifying the CP gene except that the annealing temperature was 58 °C.

Full-length DNA-A and DNA-B of ACMV/CM and EACMV/CM of a mixed-infected sample were amplified by using abutting primers (Table 1) designed from published sequences of cassava mosaic viruses or from sequenced segments reported here. The PCR mixture was as described above and the conditions of amplification were: 4 min at 94 °C and then 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 2 min at 72 °C followed by a final extension period of 10 min at 72 °C.

■ **Sequence determination and analysis.** Full-length DNA PCR products were recovered after electrophoresis in a 1% agarose gel and purified with the BIO 101 Geneclean kit. The products were digested with appropriate restriction enzymes and cloned into pBluescript II KS(+) (Stratagene). ACMV/CM DNA-A was cloned into pCR II, isolated after digestion with *Eco*RI and cloned into pBluescript II KS(+). After multiplication in *Escherichia coli* strain TOP10F′ (Invitrogen), plasmids were purified by using the Qiagen DNA purification kit. Both strands of DNA were sequenced after subcloning and/or the use of specific primers by using the ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (ABI/Advanced Biotechnology). Nucleotide and amino acid sequences were assembled and analysed with the DNASTAR package.

The source and GenBank accession numbers of geminivirus DNA-A and -B sequences used in this paper were: ACMV/KE (J02057, J02058); ACMV/NG (X17095, X17096); Althea rosea enation virus (AREV) (AF014881); chayote mosaic virus (ChaMV) (AJ223191); cotton leaf curl virus from Pakistan (CLCuV-PK1/Fai1) (AJ222703); EACMV/KE (AJ006458); EACMV/MW [AJ006461 (CP)]; EACMV/MW/K (AJ006460); EACMV/MW/MH (AJ006459); EACMV/TZ (Z83256); EACMV/UG//1 (Z83257); EACMV/UG//Svr [AF126807 (DNA-B)]; ICMV (Z24758, Z24759); potato yellow mosaic virus from Trinidad and Tobago (PYMV/TT) (AF039032, AF039033) and from Venezuela (PYMV/Ve) (D00940, D00941); and tomato yellow leaf curl virus from Israel (TYLCV-IL) (X15656) and from Sardinia (TYLCV-Sar) (X61153).

■ **Determination of synergism between ACMV/CM and EACMV/CM.** Cassava plants infected by ACMV/CM and EACMV/CM manifested unusually severe symptoms. In order to determine the occurrence of a synergistic interaction between the two virus species, infected cassava samples and cloned viral DNA were used to inoculate 21-day-old seedlings of *Nicotiana benthamiana*.

(i) Inoculation with plant sap. Cassava samples infected by ACMV/CM or EACMV/CM alone or by both viruses were used to inoculate five *N. benthamiana* seedlings mechanically. A fourth treatment was obtained by mixing sap from the same samples infected by ACMV/CM and EACMV/CM alone to obtain a doubly infected sample. Sap was obtained from the leaves of the plants at a dilution of 1:10 (w/v) in a 0·1 M phosphate buffer, pH 7·0. Infected plants were grown in the

Table 1. Primers used to amplify viral DNA from cassava samples collected in Cameroon

Primer	Sequence	Virus species	Strand	Target
JSP001	ATGTCGAAGCGACCAGGAGAT	ACMV/EACMV	Virion-sense	CP
JSP002	TGTTTATTAATTGCCAATACT	ACMV	Complementary	CP
JSP003	CCTTTATTAATTTGTCACTGC	EACMV	Complementary	CP
CR01	CTAGCTGCAGCATATTTACRARWATGCC	EACMV	Virion-sense	BC1/ICR
CR02	ACCTCTGCAGCARTGRTCCKATYTTCATAC	EACMV	Complementary	BC1/ICR
VNF003	CCCAAGCTTTGGTTAGAGGTTT	EACMV	Virion-sense	DNA-A
VNF004	CCCAAGCTTGTTCCTTCATCCCWA	EACMV	Complementary	DNA-A
VNF007	GGCCTAGGGGCTGTGAAGGYCCCA	ACMV	Virion-sense	DNA-A
VNF008	GGCCTAGGTATGTCTGGGCTTC	ACMV	Complementary	DNA-A
EB03	GTTAACATTTATTTTTTGTTTMTCGC	EACMV	Virion-sense	DNA-B
EB04	GTTAACGAAATAAAAGYWGAACGT	EACMV	Complementary	DNA-B
VNF021	CTCATCGATATAGGGTATTGC	ACMV	Virion-sense	DNA-B
VNF022	CTCATCGATGCTGTTGATTAT	ACMV	Complementary	DNA-B
VNF031	GGATACAGATAGGGTTCCCAC	EACMV/CM	Virion-sense	AC2/AC3
VNF032	GACGAGGACAAGAATTCCAAT	EACMV/CM	Complementary	AC2/AC3
VNF033	CCGTAACTTGGAGAGTGTTTA	EACMV/TZ	Virion-sense	AC2/AC3
VNF034	GGATGAGGAAAAGAATCAGTC	EACMV/TZ	Complementary	AC2/AC3

growth chamber at 24 °C and 14 h photoperiod and the symptoms were noted.

(ii) Construction of infectious clones and inoculation of *N. benthamiana*. Total DNA was extracted from leaf tissue of the *N. benthamiana* plants inoculated with ACMV/CM and EACMV/CM. Supercoiled DNA was separated from other nucleic acids on a 1·2% agarose gel. The fragments between 1·6 and 2·0 kb apparent length were isolated, cleaved with *Bam*HI and *Hin*dIII and then cloned into pUC18. Restriction mapping and sequence comparison established that clones pVF.AA, pVF.AB and pVF.EA contained ACMV DNA-A, ACMV DNA-B and EACMV DNA-A (referred to as AA, AB and EA), respectively. Head-to-tail partial repeats of these clones were constructed as described by von Arnim & Stanley (1992). EACMV/CM DNA-B could not be cloned directly and was amplified by using primers EB03 and EB04 (Table 1). A partial repeat of EACMV DNA-B (EB) could not be constructed because the *Hpa*I site used to clone it occurs three times in the sequence; therefore a monomer was used in the inoculation experiments.

Three combinations of cloned DNAs were inoculated to *N. benthamiana* mechanically (2 µg per plant) and by the biolistic method (200 ng per plant) as described by Gilbertson *et al*. (1991). The combinations were AA + AB, EA + EB and AA + AB + EA + EB.

In order to quantify and compare accumulation of viral DNAs in the singly and doubly infected plants, total DNA was extracted from inoculated *N. benthamiana* plants as described by Dellaporta *et al*. (1983). DNA was further deproteinized by phenol–chloroform extraction followed by extraction with chloroform alone. The DNA (5 or 10 µg per well) was separated on ethidium bromide-stained, 1·2% agarose gels in 1 × TAE buffer and blotted onto Hybond-N^+ membranes (Amersham).

The probes used for ACMV/CM were the fragments *Eco*RI–*Bam*HI (nt 1714–140) for DNA-A and *Bam*HI–*Eco*RV (nt 1333–2402) for DNA-B. For EACMV/CM, the fragments were *Hin*dIII–*Eco*RI (nt 924–1684) for DNA-A and a 700 bp fragment covering the region from the intercistronic region (ICR) to the 5′ terminus of BC1 from cloned PCR products for EACMV/CM DNA-B. The nucleotides are numbered from the nucleotide A* of the TAATATTA*C nonanucleotide of each sequence. The probes were labelled with [^{32}P]dATP by random priming, as described by Sambrook *et al*. (1989). The intensity of bands was quantified by using a Bio-Rad phosphorimager.

Results

Symptom expression on cassava

The south-western region of Cameroon yielded plants with unusually severe and completely systemic symptoms (occasionally resulting in defoliation) in the same field as mildly affected and incompletely systemic mosaic plants (Fig. 1*a*, *b*). Plants with severely or mildly affected stems growing from the same stem-cutting were observed. PCR diagnosis showed that severely affected plants and stems were infected by both ACMV/CM and EACMV/CM, while mildly affected ones were infected by ACMV/CM alone. The variation of symptom severity and degree of systemic infection was also observed on cassava plants in the growth chamber (Fig. 1).

PCR diagnosis

Primers specific to a ∼ 770 bp fragment corresponding to the CP gene were used to determine the presence of ACMV and EACMV in 50 stem-cutting samples collected from the field in Cameroon. All 50 samples were found to be infected by ACMV and eight, from the south-western rainforest region, were also infected by EACMV.

Analysis of the complete nucleotide sequences of DNA-A and -B of ACMV/CM and EACMV/CM

The complete nucleotide sequences of clones pVF-AA5 (ACMV/CM DNA-A), pVF-EA6 (EACMV/CM DNA-A),

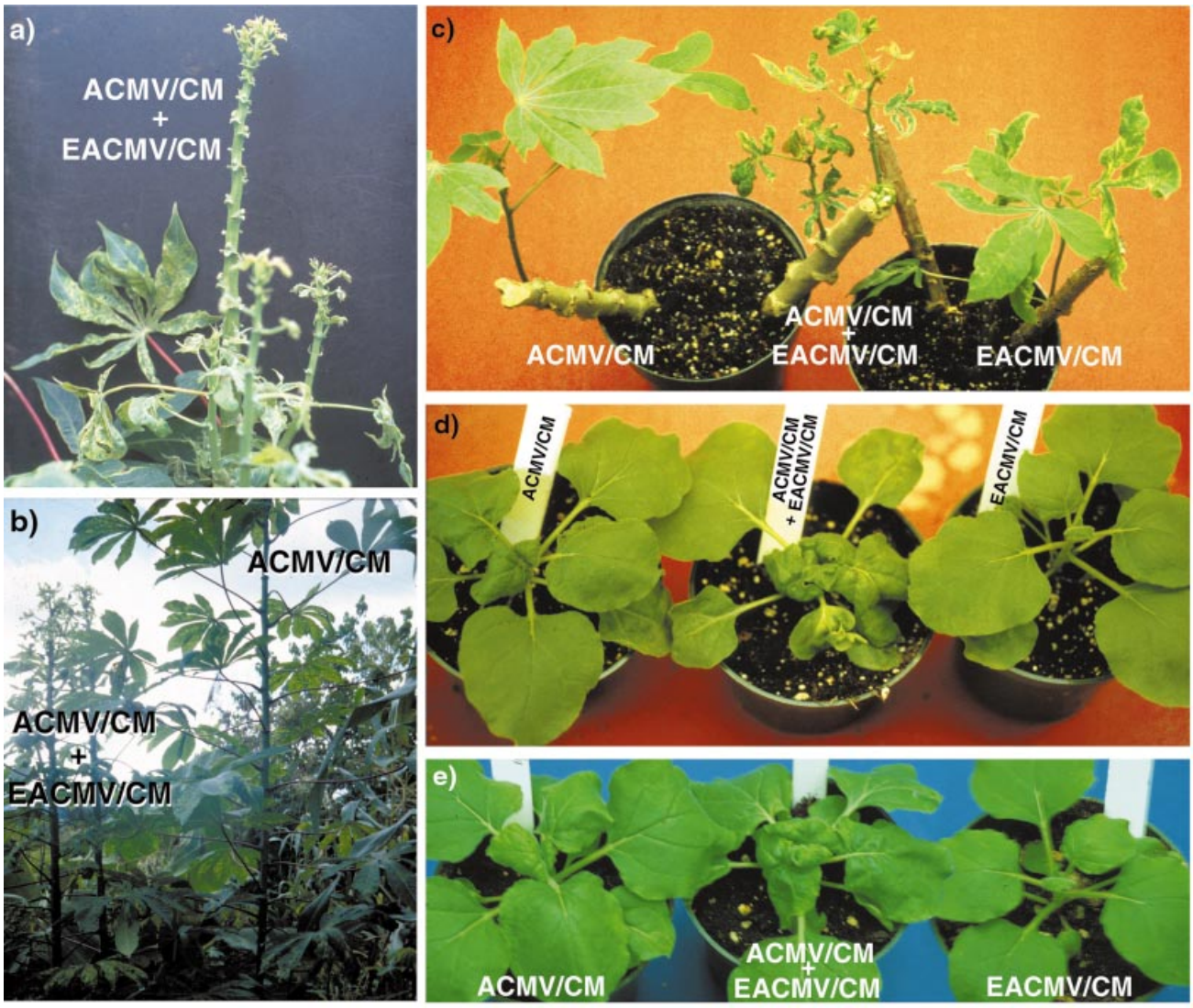


Fig. 1. (*a*) Symptoms of CMD on a cassava plant doubly infected by ACMV/CM and EACMV/CM in the south-western rainforest region of Cameroon. In such severe cases there is defoliation of the stem shoot. (*b*) In the main epidemic area, severely affected plants resulting from dual infection by ACMV/CM and EACMV/CM can be found growing next to mildly affected plants that result from infection by ACMV/CM alone. (*c*)–(*e*) The differences in symptom severity between doubly infected cassava plants and those singly infected by ACMV/CM or EACMV/CM are also observed in the greenhouse (*c*), when inoculated mechanically on *N. benthamiana* with sap from infected cassava plants (*d*) and when inoculated mechanically with cloned components of ACMV/CM and EACMV/CM (*e*). In (*c*)–(*e*), plants infected with ACMV/CM are on the left, with EACMV/CM on the right and doubly infected plants in the centre.

pVF-AB1 (ACMV/CM DNA-B) and pVF-EB1 (EACMV/CM DNA-B) were determined. The arrangement of the ORFs in each molecule was similar to that of Old World begomoviruses.

A comparison of the nucleotide sequences of both components of the two viruses and their common regions is shown in Table 2. ACMV/CM DNA-A was nearly identical (96–97%) to that of ACMV/KE and ACMV/NG, whereas EACMV/CM exhibited 84, 81 and 76% sequence identity to EACMV/TZ, EACMV/UG//1 and EACMV/MW/K, respectively. A fragment of approximately 900 nucleotides (30% of the DNA-A genome), comprising AC2 and AC3 of EACMV/CM, was very different from EACMV/TZ. This segment had 69% identity to the corresponding segment of EACMV/TZ, whereas the remainder of DNA-A exhibited 90% identity between the two viruses.

The B component sequences of ACMV/CM and EACMV/CM were aligned with those of Old World begomoviruses. The B component of ACMV/CM was highly identical to ACMV/NG (92%) and ACMV/KE (91%) and less identical to other bipartite, whitefly-transmitted geminiviruses (Table 2). Conversely, there was low sequence identity between DNA-B of EACMV/CM and EACMV/UG//Svr, a

Table 2. Comparison of nucleotide and deduced amino acid sequences of ACMV/CM and EACMV/CM ORFs with those of 13 Old World geminiviruses

Percentage nucleic acid sequence identities and amino acid similarities are shown. The accession numbers for the sequences are indicated in Methods. Values over 80% are highlighted in bold.

	Nucleotide sequences				Amino acid sequences							
Virus isolate	A	B	ICRA*	ICRB*	AV1	AV2	AC1	AC2	AC3	AC4	BC1	BV1
Identity/similarity to ACMV/CM												
ACMV/NG	**97**	**92**	**97**	**94**	**97**	**97**	**98**	**94**	**89**	**92**	**97**	**93**
ACMV/KE	**96**	**91**	**96**	**91**	**97**	**94**	**96**	**92**	**84**	**90**	**97**	**93**
EACMV/CM	60	33	–	–	73	68	68	56	56	30	53	35
EACMV/TZ	63	–	44	–	72	54	68	63	64	32	–	–
EACMV/UG//1	68	–	44	–	**89**	68	68	62	65	32	–	–
EACMV/UG//Svr	–	34	–	44	–	–	–	–	–	–	53	36
EACMV/MW//K	65	–	45	–	80	79	68	63	64	26	–	–
SACMV	–	–	42	–	71	–	–	–	–	–	–	–
ICMV	62	28	44	43	74	72	72	59	59	49	32	24
AREV	64	–	39	–	80	60	77	54	52	55	–	–
CLCuV-PK1/Fai1	63	–	43	–	72	–	75	57	56	49	–	–
ChaMV	60	–	38	–	77	–	69	52	62	–	–	–
TYLCV-IL	67	–	44	–	80	75	72	63	67	38	–	–
TYLCV-Sar	64	–	38	–	74	71	74	61	61	45	–	–
Identity/similarity to EACMV/CM												
ACMV/CM	60	33	43	41	66	72	68	55	55	29	53	38
ACMV/NG	60	32	44	44	67	52	69	58	60	30	55	35
ACMV/KE	60	32	42	45	72	54	69	59	59	28	54	34
EACMV/TZ	**84**	–	**90**	–	**94**	**93**	**91**	58	65	**90**	–	–
EACMV/UG//1	**81**	–	**99**	–	**81**	**92**	**91**	65	65	**91**	–	–
EACMV/UG//Svr	–	63	–	64	–	–	–	–	–	–	**85**	64
EACMV/MW//K	76	–	**93**	–	**91**	62	**91**	56	58	**86**	–	–
SACMV	–	–	64	–	75	–	–	–	–	–	–	–
ICMV	59	28	45	46	77	55	68	59	61	44	40	24
AREV	67	–	36	–	**83**	47	73	49	51	41	–	–
CLCuV-PK1/Fai1	64	–	42	–	76	46	75	55	58	49	–	–
ChaMV	59	–	42	–	76	–	74	52	62	–	–	–
TYLCV-IL	53	–	46	–	**81**	54	74	49	59	46	–	–
TYLCV-Sar	61	–	59	–	**83**	56	71	55	56	49	–	–

* ICRA and ICRB refer to CR sequences and sequences of about 150–170 nucleotides corresponding to the CRs of the bipartite geminiviruses shown.

new geminivirus infecting cassava in Uganda (63%) (Pita *et al.*, 1999). However, a region of approximately 500 nucleotides in BC1 showed high sequence identity (87%) to the corresponding region of the DNA-B isolated in Uganda, the remainder (80% of the genome) of the B component showing 43% sequence identity.

Sequence homologies of ACMV/CM- and EACMV/CM-encoded proteins

A complete sequence analysis of virus-encoded products is presented in Table 2. There was considerable variation between the amino acid sequence of the CP of EACMV/CM and those of other Old World begomoviruses. The EACMV/CM CP amino acid sequence showed very high similarity to those of EACMV/TZ (94%) and an EACMV from Malawi (95%) (EACMV/MW), but less similarity to those of SACMV (75%), EACMV/MW//K (87%) and ACMV (66–72%). In contrast, there was high amino acid sequence similarity (97%) between the CP of ACMV/CM and the other ACMV CP sequences cited in this paper.

As is the case for the CP, the replicase (Rep) sequence is also very conserved within a virus species. The Rep sequence was highly conserved between EACMV/CM, EACMV/TZ and EACMV/UG and EACMV/MW//K (91%), but not between EACMV/CM and CLCuV-PK1/Fai1 (75%) or ACMV (68–69%). The Rep sequences of ACMV/CM and those of other ACMVs were nearly identical (96–98%) compared with EACMV sequences (68%).

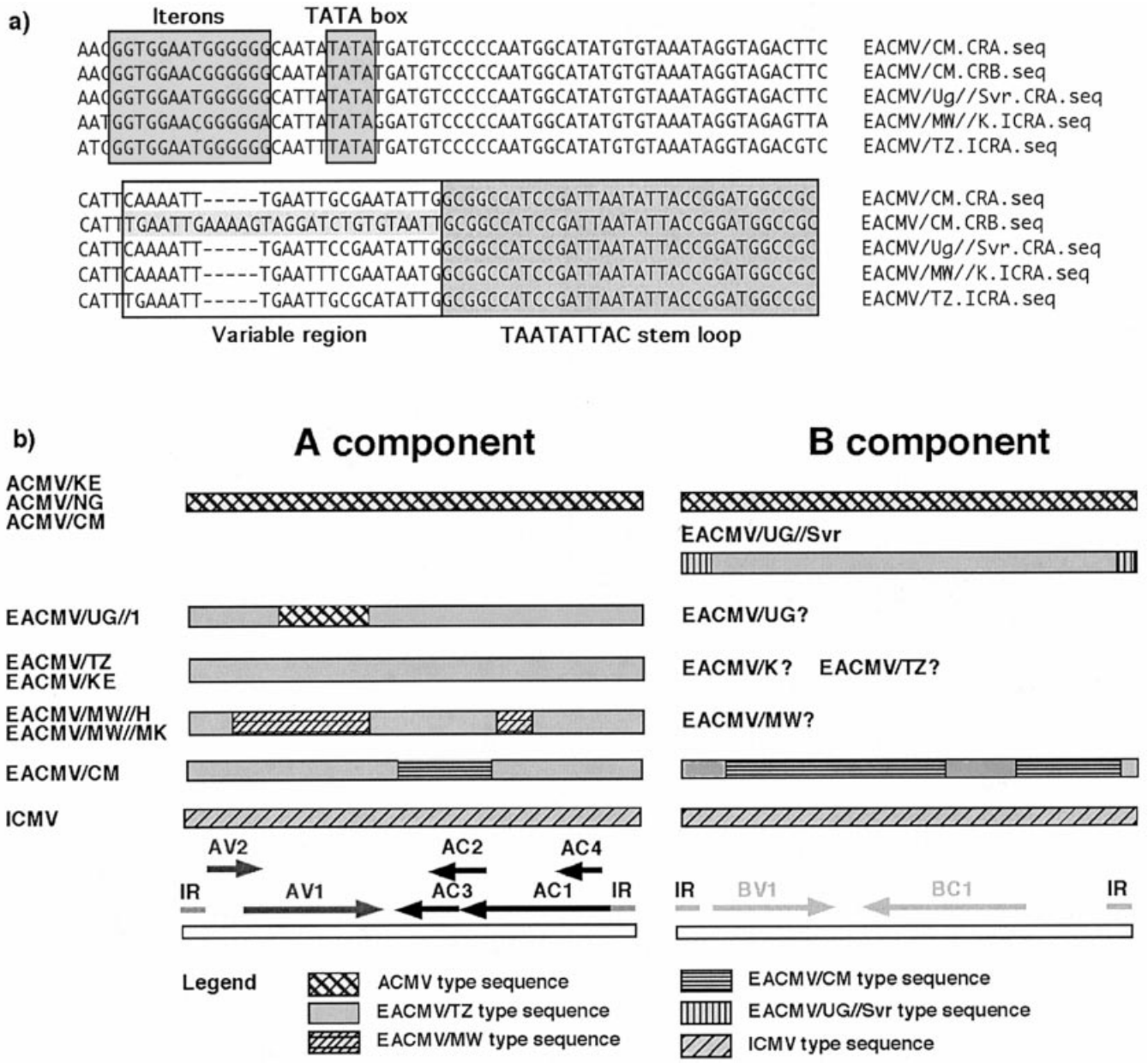


Fig. 2. (*a*) Alignment of a portion of the CRs of EACMV/CM DNA-A and DNA-B (AF112354, AF112355) and representation of the Iteron box, the TATA box and the conserved hairpin loop. The shaded area within the variable region indicates the insertion fragment in EACMV/CM DNA-B. (*b*) Genome organization and recombination maps of ACMV/KE, ACMV/NG, ACMV/CM, EACMV/TZ, EACMV/UG//1, EACMV/UG//Svr, EACMV/CM, EACMV/MW//H, EACMV/MW//MK and ICMV. Sections with similar shading represent similarity between viruses whereas different shadings denote sequence differences at the species level.

Sequence similarities between the gene regulation proteins of EACMV/CM, AC2 and AC3, and those of other EACMV were low (58–65%), and were comparable to the similarity of the corresponding sequences between EACMV/CM and isolates of ACMV (55–60%). This low value indicates that AC2 and AC3 are part of the 900 nucleotide fragment of EACMV/CM that is probably a result of a recombination with an as yet undetermined virus species. No sequence from the database with significant homology to this fragment is yet available. Sequence comparisons showed that AC4 was the least-conserved gene, with similarities between the different virus species ranging from 28 to 55% (Table 2).

To determine the homogeneity of distribution of EACMV/CM, two pairs of primers were designed from the unrelated sequence and from the corresponding region of EACMV/TZ (Table 1). The PCR results showed that all eight samples infected by ACMV/CM and EACMV/CM contained a similar fragment (data not shown). In contrast, primers

specific to EACMV/TZ for the same region did not amplify the corresponding fragment in any of the eight samples, indicating the absence of EACMV/TZ.

The two B-component proteins, BV1 and BC1, were highly conserved among the three ACMV isolates (97 and 93% similarity, respectively). In contrast, there was very low similarity (53%) between BC1 of ACMV/CM and that of EACMV/CM (Table 2).

The common regions (CRs) of ACMV/CM and EACMV/CM

Sequence identities between the CRs of ACMV/CM and other ACMV isolates were high: there was 91–96% identity to ACMV/KE and 94–97% identity to ACMV/NG, compared with 42–45% to EACMV, ICMV and SACMV isolates (Table 2). The CR of the DNA-A of EACMV/CM also showed high sequence identity to ICRs from EACMV/UG (99%) and EACMV/TZ (90%) and intermediate identity to SACMV (64%) and TYLCV-IL (59%), but very low identity to all the other viruses including ACMVs.

The CRs of the two components of ACMV/CM were 170 nucleotides long with a sequence identity of 92%, while those of EACMV/CM were 150 nucleotides long with only 80% identity. The comparatively low sequence identity in the CRs of EACMV DNA-A and -B is due to a 28 nucleotide segment of EACMV DNA-B that appears to be a deletion of 23 nucleotides and an insertion of 28 nucleotides at the 5′ end of the conserved hairpin-loop motif (Fig. 2*a*). When the 28 nucleotide sequence was not considered, the two CR sequences were 93% identical. In addition to the conserved 30-mer of the hairpin region, common to all geminiviruses sequenced to date, the putative Rep-binding site motifs upstream of the TATA box were GGTGGAATGGGGG for both components of EACMV/CM (Fig. 2*a*). This motif is similar to that reported by Zhou *et al*. (1998*b*) in EACMV isolates from eastern Africa, but is different from the motifs of EACMV/MW//MH (GGGGGAACGGGGG) (AJ006459) and SACMV (GGGGGGATGGGGG). In ACMV/CM, as in other ACMV isolates cited in this article, the repeated motif in both components was TGGAGACA.

Synergism between ACMV/CM and EACMV/CM

Cassava plants co-infected by ACMV/CM and EACMV/CM showed unusually severe symptoms under field conditions and in the growth chamber compared with singly infected plants. This suggested a synergistic interaction between the two viruses (Fig. 1*a*). In order to determine the occurrence of synergism at the level of viral DNA accumulation, *N. benthamiana* plants were inoculated with sap from cassava samples singly infected by ACMV/CM or EACMV/CM, with a mixture of sap from both samples and with sap from a naturally co-infected cassava plant. Plants co-infected with ACMV/CM and EACMV/CM presented a characteristic stunting with short internodes. They remained severely affected throughout the period of observation, especially when the plants were inoculated with sap from infected cassava leaves (Fig. 1*d*).

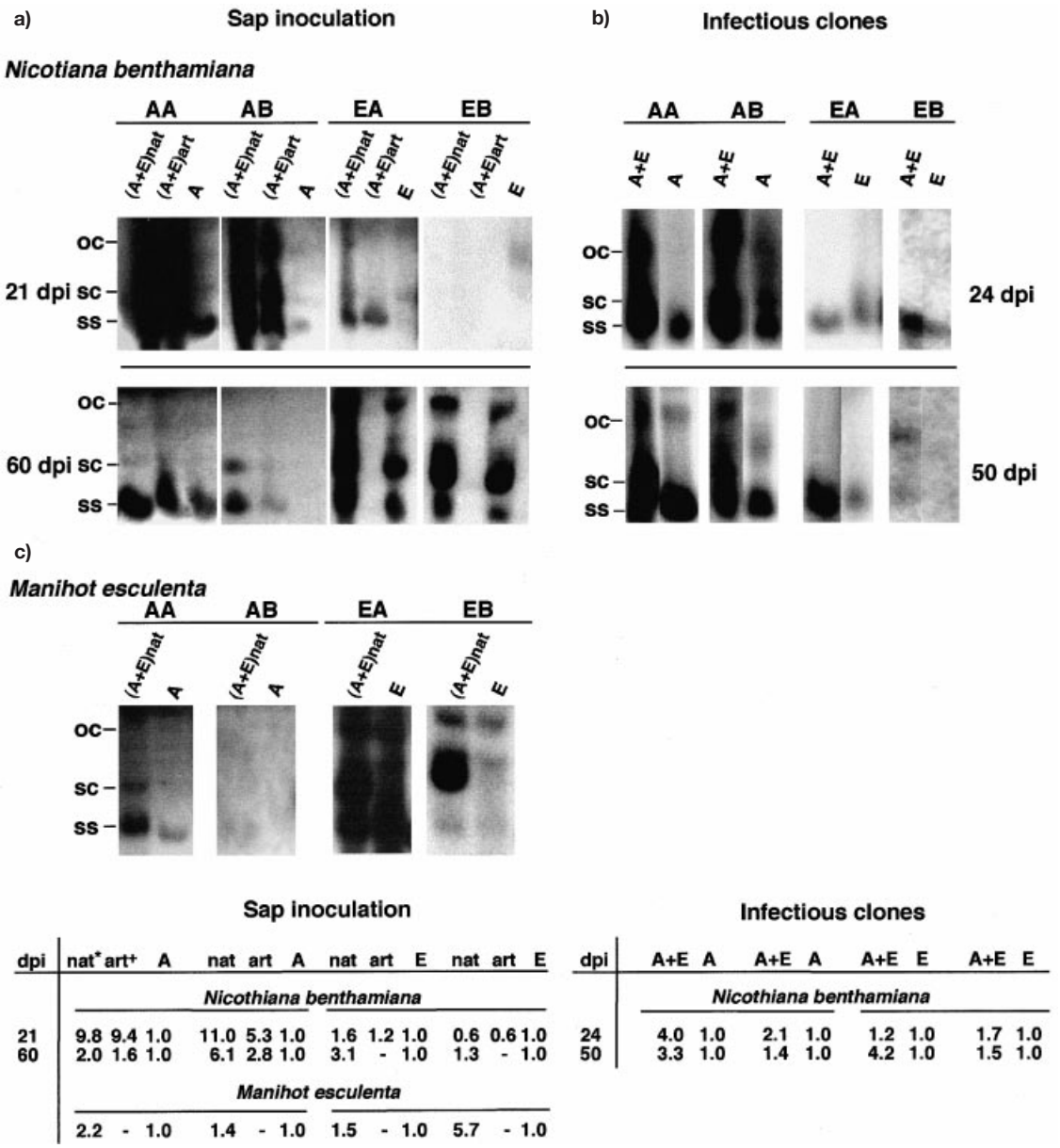
Table 3. Infectivity of genomic A and B component combinations inoculated mechanically and by the biolistic method on *N. benthamiana*

The number of plants showing symptoms is given as plants showing symptoms/plants inoculated. Severity of symptoms was scored from −, no symptoms, to + + + + +, severe symptoms.

Inoculum	Plants showing symptoms		Symptom severity
	Mechanical inoculation	Biolistic inoculation	
AA + AB	5/5	5/5	+ + +
EA + EB	0/5	0/5	−
AA + AB + EA + EB	5/5	5/5	+ + + + +

To confirm that the synergism observed was due to co-infection by ACMV/CM and EACMV/CM, cloned components of these virus species were inoculated to *N. benthamiana* plants. As observed in sap inoculations, plants inoculated with both components of the two virus species, i.e. AA + AB + EA + EB, displayed more severe symptoms than plants inoculated with the two components of either virus alone (Fig. 1*e*; Table 3). Although symptoms were not observable on plants inoculated with cloned components of EACMV/CM, Southern blot analyses detected low levels of both components in the inoculated plants (Fig. 3*b*).

To investigate the occurrence of synergism at the level of viral DNA accumulation from cloned components, Southern blot analyses were conducted with DNA from pooled leaf samples of all five plants inoculated with sap, as well as all five plants inoculated mechanically with AA + AB, EA + EB and AA + AB + EA + EB. With probes specific to each of the four DNA components, Southern blot analyses showed a higher accumulation of DNA-A and DNA-B of ACMV/CM at 21 ('sap inoculation'), 30 (data not shown) and 60 ('sap inoculation') days post-inoculation (p.i.), both with natural and artificial mixtures of the viruses infecting *N. benthamiana* plants, compared with plants infected with ACMV/CM alone (Fig. 3*a*). At 21 days p.i., the greatest increase was observed for DNA-A, with 9·8- and 9·4-fold increases, and for DNA-B, with 11·0- and 5·3-fold increases for natural and artificial mixtures, respectively. At 60 days p.i., the levels of DNA-A and DNA-B in both categories of doubly infected plants were higher than in singly infected plants (2·0- and 6·1-fold), but were lower than at 21 days p.i. in all the treatments (Fig. 3*a*, panels AA and AB). In each case, there was a slightly higher level of viral DNA in plants infected with a natural mixture of ACMV/CM



Sap inoculation

dpi	nat*	art+	A	nat	art	A	nat	art	E	nat	art	E
	Nicothiana benthamiana											
21	9.8	9.4	1.0	11.0	5.3	1.0	1.6	1.2	1.0	0.6	0.6	1.0
60	2.0	1.6	1.0	6.1	2.8	1.0	3.1	-	1.0	1.3	-	1.0
	Manihot esculenta											
	2.2	-	1.0	1.4	-	1.0	1.5	-	1.0	5.7	-	1.0

Infectious clones

dpi	A+E	A	A+E	A	A+E	E	A+E	E
	Nicothiana benthamiana							
24	4.0	1.0	2.1	1.0	1.2	1.0	1.7	1.0
50	3.3	1.0	1.4	1.0	4.2	1.0	1.5	1.0

Fig. 3. (*a*) Southern blot analysis showing levels of accumulation of DNA-A and -B of ACMV (probes AA and AB, respectively) and EACMV (probes EA and EB, respectively). Each lane contained 10 μg total DNA extracted from cassava (*M. esculenta*) or *N. benthamiana* samples (five plants pooled), singly infected by ACMV/CM (A) or EACMV/CM (E), naturally doubly infected by ACMV/CM and EACMV/CM [(A+E) nat] or artificially doubly infected by ACMV/CM and EACMV/CM [(A+E) art]. oc, Open circles; sc, supercoiled; ss, single stranded; dpi, days p.i. (*b*) Viral DNA accumulation from pooled leaves of plants inoculated mechanically with cloned A and B components of ACMV/CM and EACMV/CM. Probes were labelled with [^{32}P]dATP by random priming as described by Sambrook *et al.* (1989). (*c*) Viral DNA accumulation in cassava leaves infected by ACMV/CM and EACMV/CM. The tables show the comparative levels of DNA accumulation. The values are ratios of natural (nat) or artificial (art) double infection over single infection relative to each treatment for each probe. The pictures represent scanned images of the Southern blots and cannot be compared accurately from one treatment to the next. dpi, Days p.i.

and EACMV/CM compared with the artificial mixture. Analyses of *N. benthamiana* inoculated with cloned components of viral DNA showed a similar pattern of DNA accumulation to that in plants inoculated with plant sap (Fig. 3 *b*). With cloned DNAs there was an increase in ACMV DNA-A of 3·5- and 2·7-fold at 24 and 50 days p.i., respectively, and a smaller increase for ACMV/CM DNA-B of 1·8- and 2·3-fold at 24 and 50 days p.i. (Fig. 3 *b*; lanes AA and AB). There were considerably low levels of both EA and EB than were observed in plants inoculated with sap.

Unlike ACMV/CM, the accumulation of EACMV/CM DNA-A was similar or only slightly increased in doubly infected *N. benthamiana* plants compared with singly infected plants at 21 and 60 days p.i. (1·6- and 3·1-fold increases,

respectively) (Fig. 3*a*, lanes EA). DNA-B accumulation was lower or similar in mixed-infected plants (Fig. 3*a*; lanes EB). With cloned DNAs, we observed a similar trend but with a smaller increase for EACMV/CM; 1·2- and 3·8-fold, respectively, for 24 and 50 days p.i. The accumulation of EACMV/CM component B was barely detectable on the blots.

The results of Southern blot analyses conducted with total DNA from infected cassava plants showed that there were higher levels of accumulation of ACMV/CM DNA-A (2·2-fold) and, to a lesser extent, of DNA-B (1·4-fold) in doubly infected cassava plants compared with singly infected plants. The accumulation of EACMV/CM was very high for both DNA components compared with ACMV/CM and the increase in doubly infected plants was greatest for component B (5·7-fold) (Fig. 3*c*; lanes EA and EB).

Discussion

Recently, Fondong *et al*. (1998) reported the occurrence of ACMV and EACMV in cassava samples collected in Cameroon on the basis of CP sequence comparisons. In this article, we report the complete sequences of ACMV/CM and EACMV/CM DNA-A and DNA-B from Cameroon. This is the first report of a DNA-B component for an EACMV-like virus.

The EACMV/CM DNA-A component is about 84% identical to the EACMV DNA-A components from East Africa cited in this paper and, therefore, could be considered to be a strain of EACMV with a recombinant fragment of 900 nucleotides from an as yet undetermined virus. However, only 20% of the DNA-B sequence, located in the BC1 gene, identified in cassava mosaic virus-infected samples from Uganda (EACMV/UG//Svr; AF126807) was homologous to EACMV/CM DNA-B. The rest of the sequence, with the exception of *cis*-elements in the 150 nucleotide CR, was unrelated to EACMV/UG//Svr DNA-B.

Evidence that the B component reported in this paper belongs to EACMV/CM is provided by the fact that there was a common region between EACMV/CM DNA-A and DNA-B with 80% sequence identity. Although sequence similarity between the two components is comparatively low, they have the same *cis*-elements in their CRs and the differences consist of a substitution between the TATA boxes and the hairpin loops (Fig. 2*a*). The same differences between the two CRs were also detected in a similar virus cloned in the Ivory Coast (EACMV/CM//IC; J. S. Pita, unpublished results). Moreover, all of the cassava samples in which EACMV/CM DNA-A was detected also contained the same DNA-B component, but it was absent from the samples infected by ACMV/CM alone, stressing that this B component belongs to EACMV/CM.

Evidence that the two components of EACMV/CM resulted from recombination events was obtained from the GENECONV program. This program finds high-scoring aligned segment pairs of sequences for the detection of recombination in geminiviruses (Padidam *et al*., 1999). Recombination events in EACMV/CM are located in the AC2–AC3 and BC1 coding sequences and are therefore different from the recombination within the CP sequence in EACMV/UG (Zhou *et al*., 1997; Deng *et al*., 1997).

Zhou *et al*. (1998*a*) reported recombinations between cotton leaf curl viruses and a geminivirus isolated from okra (CLCuV-Ok; AJ002459). Umaharan *et al*. (1998) also reported recombinations in the two components of PYMV/TT, between PYMV/Ve and an unknown virus. There is also evidence that beet curly top virus (BCTV), from the genus *Curtovirus*, might have resulted from recombination between two species belonging to two different genera, a leafhopper-transmitted mastrevirus and a whitefly-transmitted begomovirus. The BCTV CP has similarities to those of the mastreviruses, whereas the rest of the genome is begomovirus-like in sequence (Stanley *et al*., 1986; Padidam *et al*., 1995, 1999).

The data suggest that EACMV/CM is a mosaic of DNA fragments originating from at least two different species (Fig. 3*b*). Since the sequences of B components from other EACMV isolates from East Africa have not yet been determined, we cannot justifiably classify EACMV/CM as a separate virus species. For now, it can appropriately be classified as a virus related by sequence to EACMV strains from East Africa. Once the B component sequences of most EACMV strains from East Africa have been determined, we will revisit the taxonomic status of this virus. It appears that geminiviruses are generated by recombination of stretches of DNA originating from different viruses present in the same hosts (Fig. 3*b*). So far, it seems that a recombination event in DNA-A always leads to another recombination event in the corresponding B component, the driving force being the necessity to have the same *cis*-elements necessary for replication of the DNA-B component. This is the case for PYMV/TT, PYMV/Ve and PYMV from Panama (PYMV/Pa; Y15034) (Umaharan *et al*., 1998). EACMV/CM is the first case of a virus with most of its DNA-A and DNA-B probably originating from two different virus species; it is therefore a true *trans*-complement hybrid.

In this article, we report the first evidence of synergism between two geminiviruses that results in an increase in viral DNA and symptom severity. Southern blot analyses indicated that doubly infected cassava and *N. benthamiana* plants had higher levels of accumulation of DNA-A and -B of ACMV/CM during the early stages of infection, compared with plants infected with ACMV/CM alone. The level of EACMV/CM was only higher in mixed infections, especially in late stages of infection and particularly in naturally doubly infected cassava plants. These data were confirmed by using infectious clones of ACMV/CM and EACMV/CM in *N. benthamiana*. However, there was poor infectivity of EACMV/CM in *N. benthamiana*, particularly the B component

(Fig. 3*b*; lanes EB). It is possible that, even though EACMV/CM is capable of infecting *N. benthamiana*, the latter is not an adaptable host compared with cassava (Fig. 1*c*, *d*). This is also supported by the difficulty of infecting *N. benthamiana* from other *N. benthamiana* plants (Swanson & Harrison, 1994; our unpublished observations). The poor infectivity of EACMV/CM DNA-B is probably the result of the use of a monomeric and not a dimeric clone.

This type of synergism is similar to that observed with potyviruses, which mediate the accumulation of potex-, como- and machlomoviruses, but levels of which remain relatively unchanged during mixed infections (Damirdagh & Ross, 1967; Calvert & Ghabrial, 1983; Goldberg & Brakke, 1987; Vance, 1991; Anjos *et al*., 1992; Vance *et al*., 1995). However, in the synergism between ACMV/CM and EACMV/CM, there were increases in the DNA accumulation of both viruses in mixed infections. Preliminary results suggest that there is *trans*-complementation between ACMV/CM and EACMV/CM; confirmation of these results could partly explain the synergism observed in this study.

The results of DNA hybridization correlated with symptom severity in cassava and in *N. benthamiana*, since doubly infected cassava and *N. benthamiana* showed more severe symptoms throughout the period of observation than plants infected by either virus alone (Fig. 1*b–d*).

Unlike the previous cases of synergistic infections, both ACMV/CM and EACMV/CM belong to the same genus and are transmitted by the same whitefly vector and therefore will more likely co-infect the same plant than if they were spread through different modes. Consequently, doubly infected plants have considerable potential as sources of inoculum for both viruses and whiteflies feeding on such plants would, therefore, more easily acquire and transmit both viruses to virus-free plants.

There are epidemiological implications of double infections of ACMV/CM and EACMV/CM. As indicated above, plants with mixed infections of ACMV/CM and EACMV/CM exhibited symptoms on all the leaves. In contrast, plants infected by ACMV/CM or EACMV/CM alone showed incomplete systemic infection and some leaves remained symptomless. Fargette *et al*. (1994) reported that disease-free cuttings could be recovered from symptom-free areas of cassava plants. This suggests that doubly infected plants would have a lower proportion of disease-free cuttings than singly infected plants. Moreover, we have observed that reversion is inversely proportional to symptom severity (Fondong *et al*., 2000) and, therefore, doubly infected plants will be less likely to revert than singly infected plants. However, such plants would probably not be used for cuttings, as they would be too stunted to be chosen for multiplication. The spread of EACMV/CM will therefore depend, to a large extent, on transmission by whitefly. It is obvious that an increase in viral DNA content by 3- to 11-fold will dramatically increase the percentage of transmission of the viruses by whitefly.

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