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CHAPTER 4

Characterization of a naturally-occurring truncated cassava mosaic geminivirus associated with cassava mosaic disease

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Geminivirus defective interfering (DI) DNAs arise spontaneously in mechanically inoculated test plants. We report here for the first time the cloning and characterization of a naturally-occurring truncated form of cassava mosaic geminivirus (CMG) DNA-A (1525 nts), which upon sequence analysis was shown to be a defective (df) form of *East African cassava mosaic virus* (EACMV) DNA-A that has retained its *cis* elements essential for replication by the helper virus. Phylogenetic comparisons placed the df DNA-A 15 molecule close to EACMV-UG2 mild and severe isolates (>95% nucleotide sequence identity) of EACMV. Biolistic inoculation of *Nicotiana benthamiana* with infectious df DNA-A 15 clone and *East African cassava mosaic Cameroon virus* (EACMCV) resulted in symptom amelioration as compared to the EACMCV singly-inoculated plants, and there was an accumulation of the df DNA-A 15

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in systemically infected leaves. In addition, the level of EACMV- DNA-B accumulation was reduced in the coinoculated plants compared to the plants inoculated with EACMCV alone. In contrast, inoculation of df DNA-A15 with *African cassava mosaic virus Cameroon* ACMV-[CM] (DNA-A and B) did not result in detectable levels of the df DNA-A 15 in the systemic leaves and there was no significant changes in viral DNA-Accumulation and, symptom phenotype of the helper virus. PCR and sequence analysis confirmed the helper virus to be EACMV.

4.1 INTRODUCTION

Cassava mosaic disease (CMD) is widespread throughout Africa and is the most important constraint to cassava production. CMD is caused by cassava mosaic geminiviruses (CMGs) belonging to the genus *Begomovirus* of the family *Geminiviridae.* They are transmitted by the whitefly *Bemisia tabaci* (Genn.) and spread through infected cuttings, which are the usual modes of cassava propagation. *African cassava mosaic virus* (ACMV) and *East African cassava mosaic* (EACMV) are the most commonly occurring (CMGs) (Swanson and Harrison, 1994). The CMG genome consists of two molecules of single-stranded DNA (DNA-A and DNA-B), each of about 2.8 kbp (Lazarowitz, 1992). DNA-A contains six partially overlapping open reading frames (ORFs) organized in two divergent transcriptional units separated by an intergenic region (IR). On the virion sense strand, DNA-A contains AV1 and AV2 ORFs and AC1 to AC4 are on the complementary sense strand. The DNA-A encoded gene products are replication-associated protein AC1 (Rep), AV1 coat protein (CP), and proteins that participate in the control of replication AC3 and gene expression AC2 (TrAP). DNA-B encodes proteins required for nuclear trafficking BV1 and cell-to-cell movement BC1 of the viral DNA (Hamilton *et al*., 1984; Hanley-Bowdoin *et al*., 1999). Both DNA components (DNA-A and DNA-B) share a high nucleotide identity in the intergenic region (IR) (approximately 200 nts) called the common region (CR), which contains promoter and sequence elements required for DNA replication and transcription (Lazarowitz, 1992; Eagle *et al*., 1994; Chatterji *et al*., 1999).

Small subgenomic DNA species are often associated with geminivirus infection (Stenger *et al*., 1992). They are usually derived from a partial deletion of the wild-type viral genome and thus show a high degree of sequence homology to the helper virus. These defective interfering (DI) molecules have been described for a few geminiviruses, and originate from sequence deletions of either DNA-A or B. The DIs are normally about half the size of the full length molecule and contain the origin of replication and *cis* elements required for initiation of replication and often cause alterations of normal disease progression induced by their helper viruses such as symptom attenuation (Stanley *et al*., 1990; Mansoor *et al*., 2003). Only DI derived from DNA B of CMGs has been reported and now we report for the first time results of a study undertaken to characterize a putative defective DNA fragment that was PCR-amplified from CMD-infected cassava plant from a cassava field in Tanzania. Its biological and sequence characteristics were determined.

4.2 MATERIALS AND METHODS

4.2.1 Source of viral DNA. The defective DNA was identified from a geminivirus-infected cassava plant collected in the Kagera region, northwestern, Tanzania in September 2002. Young cassava leaves as well as a ardwood cassava cutting were collected. The cassava cutting was planted in the growth chamber at the Donald Danforth Plant Science Center (DDPSC), USA and symptoms monitored regularly on the newly produced leaves for 60 days. The plant was kept at a temperature of 28°C with a day length of 16 hrs and used as a source of DNA for subsequent analysis.

4.2.2 PCR, cloning and sequencing. The subgenomic defective DNA was amplified by PCR from a nucleic acid sample obtained from a field collected CMD-infected cassava plant and from the same plant kept in a growth chamber at DDPSC. Total cellular DNA from cassava leaves was extracted by the method of Dellaporta *et al*. (1983). Initially, the universal primer pair UNIF/F (5' KSGGGTCGACGTCATCAATGACGTTRTAC 3') and UNIR (5' AARGAATTCATKGGGGCCCARARRGACTGGC 3'), where K = G+T, R = A + G, S = G + C (Invitrogen, Life Science, USA) designed to amplify near-full length DNA-A of CMGs (2.7-2.8 kbp) was used. These degenerate primers with annealing positions in the AC1 gene leave

about 17 nts to reach the viral genome full length. PCR was performed with a first cycle of 1 min at 94°C followed by 30 cycles of 1 min at 94°C, 1.5 min at 55°C and 2 min at 72°C and final extension at 72°C for 10 min. In addition to the expected full-length (2.8 kbp) product, a 1.5 kbp fragment was also amplified by the same primers. The PCR products were recovered after electrophoresis in a 1% agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen, German) following the manufacturer's instruction. The 2.8 kbp PCR products were first subjected to restriction analysis by digesting with *Eco*RV and M*lu*I and then electrophoresed on a 1% agarose gel after staining with ethidium bromide. PCR amplification with primers UV-AL1/F1 and ACMV-CP/R3 (Zhou *et al*., 1997) was used to confirm the results of the restriction analysis. Both the 2.8 and 1.5 kbp PCR products were first cloned into the TOPO plasmid vector (pCR® 2.1TOPO®) using the TA Cloning Kit (Invitrogen, Life Sciences, USA) following the manufacturer's instructions and transformed into chemically competent *Escherichia coli* strain DH5α™-T1^R. Clones were confirmed positive for viral inserts using *Eco*RI restriction digestion of the extracted plasmids and PCR primers used for initial amplification. DNA was sequenced in both orientations essentially as described by Fondong *et al.* (2000). Subsequently, abutting primer pair DI 15H/F (5′-CTCACAAGCTTACATTGAAAAGGGAGGGG-3′) and DI 15H/R (5′-GGGTCAAGCTTTGACATCGGACGATGATT) were designed around an internal *Hin*d III unique site (underlined) and used to amplify 1.5 kbp PCR products from the original sample. The PCR products were purified as described above and used for subsequent analysis. Investigation of the sequence confirmed it to be a defective DNA-A component of the CMG referred to hereafter as defective (df) DNA-A 15.

4.2.3 Sequence analysis. Nucleotide sequences were assembled with the DNASTAR package. Multiple sequences were aligned using the CLUSTAL-W option of the Mac Vector 7.2 package (Accelry, San Diego, Inc, USA). The phylogenetic trees were constructed using a neighbor-joining majority rule consensus with 1000 bootstrapped replicates. The common region sequences of DNA-A from CMGs were also assembled and analyzed in a similar manner. Sequences for alignment were obtained from the GenBank Database (Table 4.1). The

extent of ORF disruption in the df DNA-A 15 was identified by complete nucleotide sequence alignment with the closest related selected CMGs.

4.2.4 Construction of an infectious clone. A partial dimer of the df DNA-A 15 was constructed as follows; The PCR product (1.5 kbp) amplified with primers, DI 15H/F and DI 15H/R was cloned into the pGEM®-T Easy Vector (Promega, USA) to produce clone pGEM df DNA-A 15 as described by the manufucturer and the ligation mix was transformed into chemically competent *Escherichia coli* strain JM109 (Promega, USA). After an overnight multiplication at 37°C, plasmids were purified with the Qiagen DNA purification kit. An *Bam*HI-*Hin*dIII fragment (645 bp) from pGEM df DNA-A 15 containing the entire IR of df DNA-A 15 was excised and cloned into *Bam*HI/*Hind*III double-digested pBluescript® II SK + (Stratagene) generating plasmid pSKdf DNA-A 15 (645). Then a full-length (1.5 kbp) fragment was excised from pGEM df DNA-A 15 by digestion with *Hind*III and inserted into a Hind III digested pSKdf DNA-A 15 (645) to form a partial dimer pSKdf DNA-A 15 (645)(df DNA-A 15) in a tandem repeat. Restriction analysis was used to confirm the correct orientation of the inserts.

4.2.5 Infectivity assay. Infectivity of df DNA-A 15 was evaluated by biolistic inoculation of *Nicotiana benthamiana* with virus clones harboring partial dimers of ACMV-Cameroon (ACMV-[CM]), and EACMV-Cameroon (EACMCV) essentially as described previously (Swanson and Harrison, 1994; Fondong *et al*., 2000; Pita *et al*., 2001). To investigate the biological role of the df DNA-A 15 in symptom modification, *N. benthamiana* plants were biolistically inoculated with cloned DNA (100 ng per plant). The combinations were (i) ACMV-[CM] (DNA-A and DNA-B) + df DNA-A 15, (ii) EACMCV (DNA-A and DNA-B) + df DNA-A 15, and df DNA-A 15. For each combination, five plants were inoculated and two control plants were not inoculated. Inoculated plants were held in a greenhouse and disease symptoms were monitored visually for 35 days.

4.2.6 Southern blot analysis. In order to quantify and compare accumulation of viral DNAs in the inoculated plants, total DNA was extracted from inoculated *N. benthamiana* 14 days post inoculation essentially as described by Dellaporta *et al*. (1983). Original field samples

containing the df DNA-A 15 were also probed for the presence of the subgenomic molecule using the full-length DNA genome of the df DNA-A 15 in duplicate. DNA replication was assessed by Southern blot analysis of the total DNA extract. A total of 5 μg was separated on a 1% agarose gel by electrophoresis and transferred to Hybond-N^+ nylon membranes (Amersham) and hybridized to probes specific to ACMV-[CM] or EACMCV genomic components (DNA-A and B) (Fondong *et al*., 2000). The probes were labeled with [^{32}P] dATP by random priming as described by Sambrook *et al*. (1989). The probe used for the hybridization to df DNA-A 15 was a full-length 1525 nts DNA fragment of the same defective molecule excised as a monomer by *Hind* III from the clone pGEM df DNA-A 15.

4.3 RESULTS

4.3.1 Symptoms on cassava plants

In the field, cassava plant containing the df DNA-A 15 expressed moderate mosaic, leaf distortion and yellowing (Fig. 4.1a). When a cutting from the same plant was planted in the growth chamber, disease symptoms developed on the newly formed leaves from 7 days after planting and resembled those observed in the field. They consisted of yellow mosaic with moderate leaf distortion (Fig. 4.1b).

4.3.2 PCR

PCR confirmed that subgenomic df DNA-A 15 (1.5 kbp) was present in cassava leaf samples collected from a CMD-infected cassava field (Fig. 4.2a). Only one sample from a single field out of the 325 PCR-positive samples collected from a different cassava growing areas in Tanzania produced the sub-genomic fragment. Primer pair UNI/F and UNI/R in addition, amplified the 2.8 kbp expected for the full-length DNA-A component of the CMG. PCR of leaf extracts from the same plant grown in the growth chamber yielded both 1.5 and 2.8 kbp PCR products one and five months after planting using both primer pairs UNI/F/R and DI 15H/F/R.

4.3.3 Identification of the helper virus

Restriction analysis with *Eco*RV gave two DNA fragments characteristic of EACMV and yielded four fragments when digested with *Mlu*I as expected for EACMV-UG2 (Fig. 4.2b). To confirm results of the restriction analysis, PCR with primer pair UV-AL1/F1 and ACMV-CP/R3 designed specifically for amplification of EACMV-UG2 yielded the expected PCR product (results not shown). This primer amplifies DNA-A of EACMV-UG2 but not EACMV-TZ (Zhou *et al*., 1997). Partial nucleotide sequence (593 nts fragment 5′→3′ from the beginning of the UNIF primer in the AC1 gene) comparison of the helper virus (EACMV-TZ15), with corresponding fragments of df DNA-A 15, EACMV-UG2Svr (AF126806), EACMV-UG2Mld (AF126804), and EACMV-[TZ] (Z83256) showed high nucleotide sequence identity with 96%, 98%, 97% and 93% respectively.

4.3.3 Sequence analysis and comparison

4.3.3.1 Phylogenetic comparisons of df DNA-A 15 with 23 other begomoviruses infecting cassava

Begomoviruses used in comparative sequence analyses and their respective acronyms and GenBank accession numbers are shown in Table 4.1. The complete nucleotide sequence of df DNA-A 15 is available in the GenBank database under accession number AY 676464 and that of the helper virus EACMV-TZ15 is AY828226. The df DNA-A 15 clone was 1525 nts in length (55% of the DNA-A genome of other CMGs. The phylogenetic analysis based upon alignments of the complete nucleotide sequences of DNA-A components of CMGs is shown (Fig. 4.3a). The df DNA-A 15 clearly groups with EACMV species. The highest overall nucleotide sequence identity was found with EACMV-UG2Svr (96%), EACMV-Ug2Mld (95%), EACMV-UG2 (95%), EACMV-[KE-K2B] (92%), EACMV-[TZ] (91%), EACMCV-CM[IC] (88%) and EACMCV (87%) and a low identity with SLCMV-[Col] (52%). The df DNA-A 15 shows only low (< 50%) sequence homology to the ACMV isolates.

Since great similarity between the intergenic regions of two geminivirus isolates constitutes strong evidence that they are closely related, a comparison of the nucleotide sequence of df DNA-A 15 CR was made to corresponding published CR sequences of begomoviruses. The phylogenetic analysis revealed df DNA-A 15 to have high sequence similarity to EACMV-UG2Svr (97%) and EACMV-UG2Mld (96%) to which it clustered closely (Fig. 4.3b). It had nucleotide sequence identity of 80% and 78%, to EACMCV and EACMCV-CM [IC] respectively. The df DNA-A 15 differed greatly from the ACMV isolates (< 50% sequence identity).

To examine closely the nature of regulatory sequences of the df DNA-A 15, its CR (185 nts) was aligned with four EACMV isolates (Fig. 4.4). Comparison of df DNA-A 15 nucleotide sequences of the CR with those of other selected cassava begomoviruses revealed the typical motifs conserved in cassava begomovirus CR sequences. The nine-base invariant sequence (TAATATTAC) containing the Rep protein nick site was found at the 3′ region of the CR (Fig. 4.4). The sequence of the inverted repeats flanking the invariant sequence that collectively form the stem-loop structure of the *ori* was conserved in the df DNA-A 15 and resembled those of EACMCV, EACMCV-CM [IC], EACMV-UG2Svr and EACMV-UG2Mld (Fig. 4.4). The Rep-binding site motif of df DNA-A 15 upstream of the TATA box was GGTGGAATGGGGG similar to that reported for EACMCV, EACMCV-CM [IC] (Fondong *et al*., 2000) and for EACMV-UG2Svr and EACMV-UG2MLd (Pita *et al*., 2001) (Fig. 4.4).

4.3.3.2 Analysis of df DNA-A 15 ORFs

The MAPDRAW program of the DNASTAR package was used to analyse the ORFs in the df DNA-A 15. The complete sequences of two DNA-A components of EACMCV and EACMV-UG2Svr were used for size comparison with the ORFs of df DNA-A 15. On the virion-sense (V), two ORFs were identified, AV2 and AV1. The size of AV2 and AV1 were 328 and 167 nts long respectively. The C-terminal sequence of AV2 had been deleted and a large portion of the AV1 as well. On the complementary-sense (C), only two ORFs were found. The AC4,

which was 236 nts long had both the start and stop codons and was only two nucleotides larger than that of EACMCV and EACMV-UG2Svr. The AC1 was 871 nts long with only part of its C-terminal sequence deleted. Two ORFs commonly present in the DNA-A component of other CMGs, AC2 and AC3 were missing in df DNA-A 15 (Fig. 4.5).

4.3.3.3 df DNA-A 15 ameliorates symptoms of EACMCV but not of ACMV-[CM]

Nicotiana benthamiana plants biolistically coinoculated with ACMV-[CM] and df DNA-A 15 developed systemic mosaic symptoms 5 dpi that were indistinguishable from those induced by ACMV-[CM] alone (Fig. 4.1c and d) and plants recovered equally at about 21 dpi. Plants inoculated with EACMCV alone expressed severe systemic symptoms starting at 6 dpi, displaying mosaic and downward leaf curling and did not recover (Fig. 4.1e). In contrast, however, all the plants coinoculated with EACMCV and df DNA-A 15 developed only mild systemic mosaic symptoms 13 dpi (Fig. 4.1f) with very slight leaf distortion. Plants inoculated with df DNA-A 15 alone remained asymptomatic throughout the experiments (Fig. 4.1g).

4.3.4 Mediation of df DNA-A 15 systemic movement by cassava mosaic geminiviruses DNA

Southern blot analysis of total DNA collected from systemic symptomatic leaves of *N. benthamiana* inoculated with the ACMV-[CM] and df DNA-A 15 combination showed no detectable level of the subgenomic df DNA-A 15 (Fig. 4.6a). Furthermore, there was no significant change in the levels of viral DNA of both components DNA-A and DNA-B (Fig. 4.6a and b). However, df DNA-A 15 was detected in systemically infected tissue after inoculation with EACMCV but not when inoculated alone (Fig. 4.6c). Analysis of the viral DNA showed a reduced level of EACMCV DNA-B accumulation in plants inoculated with the EACMCV and df DNA-A 15 combination but not in plants inoculated with EACMCV alone (Fig. 4.6e). The df DNA-A 15 replication form was also detected in the original field sample though in a smaller amount compared to that observed in *N. benthamiana* (Fig. 4.6d).

4.4 DISCUSSION

Our study has demonstrated for the first time the occurrence in nature of a defective DNA-A of EACMV in cassava. The subgenomic DNA reported for CMGs-infected plants (Stanley and Townsend, 1985) was derived from ACMV DNA-B and was isolated from *Nicotiana benthamiana* mechanically inoculated with a Kenyan isolate of ACMV. In contrast, df DNA-A 15 was detected in nature in a CMD-infected cassava plant in the field and could be recovered from the same plant when replanted in a growth chamber to reproduce symptoms systemically.

Investigation of the sequence of df DNA-A 15 confirmed it to be a defect of EACMV DNA-A. Furthermore, the sequence confirmed the presence of IR and the 5′ part of AV1 gene and ~80% of the AC1 gene. Subgenomic defective DNA molecules, associated with a number of begomoviruses (Stanley and Townsend, 1985; MacDowell *et al*., 1986; Czosnek *et al*., 1989; Stanley *et al*., 1997) seem to be fairly uniform in structure and have tended to retain their IR and a large portion (5′ end) of AC1 ORFs as observed in this study for, in which a large portion of BV1 or CP (AV1) and remaining ORFs were shown to have been deleted.

Subgenomic single-stranded DNA molecules of about half the size of the genomic DNA have also been detected in plants infected with other begomoviruses and have high genome sequence homology with their respective helpers from which they are derived. In this study, phylogenetic analysis of the complete nucleotide sequence of df DNA-A 15 showed its close relationship to the EACMV-UG2 isolates for which the overall nucleotide sequence identity is 95-96%. A similar pattern was also observed when only the common region (CR) of the df DNA-A 15 was compared to other EACMV isolates. Partial sequence of EACMV-TZ15, the helper virus of df DNA-A 15 showed high nucleotide sequence identity to the defective molecule and other EACMV isolates suggesting that df DNA-A 15 indeed defected from EACMV.

Defective interfering molecules are also associated with many plants and nearly all animal RNA viruses (Simon *et al*., 2004) and contain all the *cis*-acting elements necessary for RNA-dependent RNA polymerases (RdRp) of the parental virus. Defective molecules are also described for the leafhopper transmitted *Beet curly top virus* (BCTV) (*Geminiviridae:*

Curtovirus); (Stenger *et al*., 1992) the genome of which contains elements similar to those in begomovirus DNA-A. Moreover, the BCTV defective molecules resemble those of *cotton leaf curl virus*–Pakistan (CLCuV-PK) in retaining the intergenic region and part of the *Rep* gene. The df DNA-A 15 was also found to retain all the regulatory sequences in the CR and a large part of the Rep protein as observed earlier (Liu *et al*., 1998). This molecule would then be replicated in substantial amounts by the normal mechanism for viral DNA-A.

The biological effect of df DNA-A 15 was determined experimentally in the greenhouse using biolistic inoculation of *N. benthamiana*. DI spontaneously produced by geminiviruses reduce the severity of the virus disease during the infection process by competing with the genomic components for cellular resources (Mansoor *et al*., 2003) suggesting that efficient replication of the DI by the helper virus is a prerequisite for the symptom amelioration to occur. In this study, df DNA-A could be replicated by EACMCV resulting in symptom attenuation and reduction in DNA-Accumulation of its cognate DNA B but not of ACMV-[CM]. The predicted replication initiator protein (Rep) binding motif for df DNA-A 15 is GGTGGAATGGGGG, similar to that for EACMCV. The df DNA-A 15 accumulated to higher levels only when coinoculated with EACMCV in *N. benthamiana* but not when inoculated alone suggesting that it was easily recognized and efficiently replicated by the EACMCV Rep. ACMV-[CM] on the other hand has a Rep binding motif repeat of TGGAGACA (Fondong *et al*., 2000), which is different from that found in df DNA-A 15 making it difficult for trans-replication to occur. Indeed we did not observe a detectable level of df DNA-A 15 in plants coinoculated with ACMV-[CM] and systemic symptom severity was indistinguishable from that induced by ACMV-[CM] alone.

It has been suggested that defective genomes gain a replication advantage over the wild type simply because they are shorter. In addition, selection favors defective genomes that outcompete the wild type for replication enzymes and capsid proteins. This competition causes coinfected cells to produce few wild-type viruses and many fully coated, infectious viruses with shortened DI genomes (Steven, 2000). The helper virus EACMCV (DNA-B) also only accumulated to a low level. Since DNA-B encodes for symptoms, low levels of DNA-B may contribute to the symptom attenuation. This was consistent with the mild symptoms observed

in all the plants inoculated with the EACMCV and df DNA-A 15 combination. Recently, new models for DI-mediated reduction in helper virus levels and symptom attenuation include DI enhancement of post-transcription gene silencing (PTGS), which is an antiviral defense mechanism in plants as demonstrated for RNA plant viruses (Simon *et al*., 2004). Whether this is true also for plant DNA viruses remains to be studied to gain insights of the relationship between subviral DNA, helper viruses and the hosts. The possibility of the truncated Rep gene of df DNA-A 15 to act as dominant negative mutant rather than acting as a defective interfering molecule, trans-encapsidation and transmission remain to be investigated to gain understanding of the mechanism of symptom modulation by this DNA mutant.

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Table 4.1. Cassava mosaic geminiviruses (DNA-A) used in comparative sequence analysis and their respective GenBank accession numbers

Name	Acronym	Acc. number
African cassava mosaic virus – [Cameroon]	ACMV-[CM]	AF112352
African cassava mosaic virus – [Cameroon- DO2]	ACMV-	AF366902
African cassava mosaic virus –[Ivory Coast]	ACMV-[IC]	AF259894
African cassava mosaic virus – [Kenya]	ACMV-[KE]	J02057
African cassava mosaic virus – [Nigeria]	ACMV-[NG]	X17095
African cassava mosaic virus – [Nigeria-Ogo]	ACMV-[Ng-	AJ427910
African cassava mosaic virus – [Uganda]	ACMV-[UG]	Z83252
African cassava mosaic virus – Uganda Mild	ACMV-	AF126800
African cassava mosaic virus – Uganda Severe	ACMV-UGSvr	AF126802
East African cassava mosaic Cameroon virus	EACMCV-CM	AF112354
East African cassava mosaic Cameroon virus -	EACMCV-CM	AF259896
East African cassava mosaic virus – [Kenya-K2B]	EACMV-KE-	AJ006458
East African cassava mosaic Malawi virus –[MH]	EACMMV-	AJ006459
East African cassava mosaic Malawi virus – [K]	EACMMV-	AJ006460
East African cassava mosaic virus – [Tanzania]	EACMV-[TZ]	Z83256
East African cassava mosaic virus – Uganda2	EACMV-UG2	Z83257
East African cassava mosaic virus – Uganda2 Mild	EACMV-	AF126804
East African cassava mosaic virus – Uganda2	EACMV-	AF126806
East African cassava mosaic Zanzibar virus	EACMZV	AF422174
East African cassava mosaic Zanzibar virus –	EACMZV-	AJ516003
Sri Lankan cassava mosaic virus-[Colombo]	SLCMV-[Col]	AF314737
South African cassava mosaic virus	SACMV	AF155806
South African cassava mosaic virus –[M12]	SACMV- [M12]	AJ422132

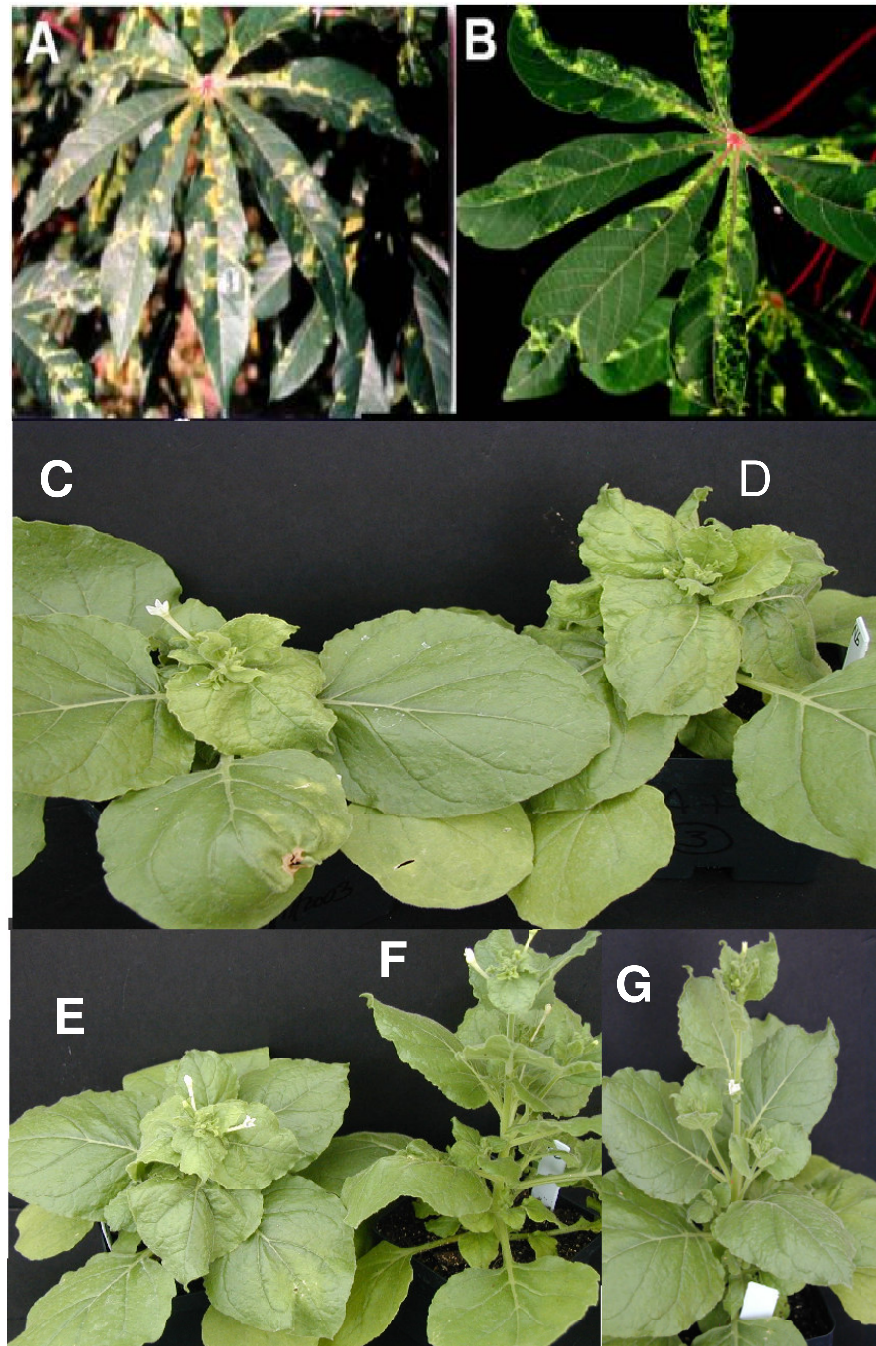

Fig. 4.1. Disease symptoms of CMG (a) on cassava field plant containing the df DNA-A 15 and (b) symptoms reproduced in the growth chamber. *Nicotiana benthamiana* plants inoculated with (c) ACMV-[CM], D) ACMV-[CM] + df DNA-A15, (e) EACMCV, (f) EACMCV + df DNA-A 15 and (g) df DNA-A 15 alone 14 days after inoculation.

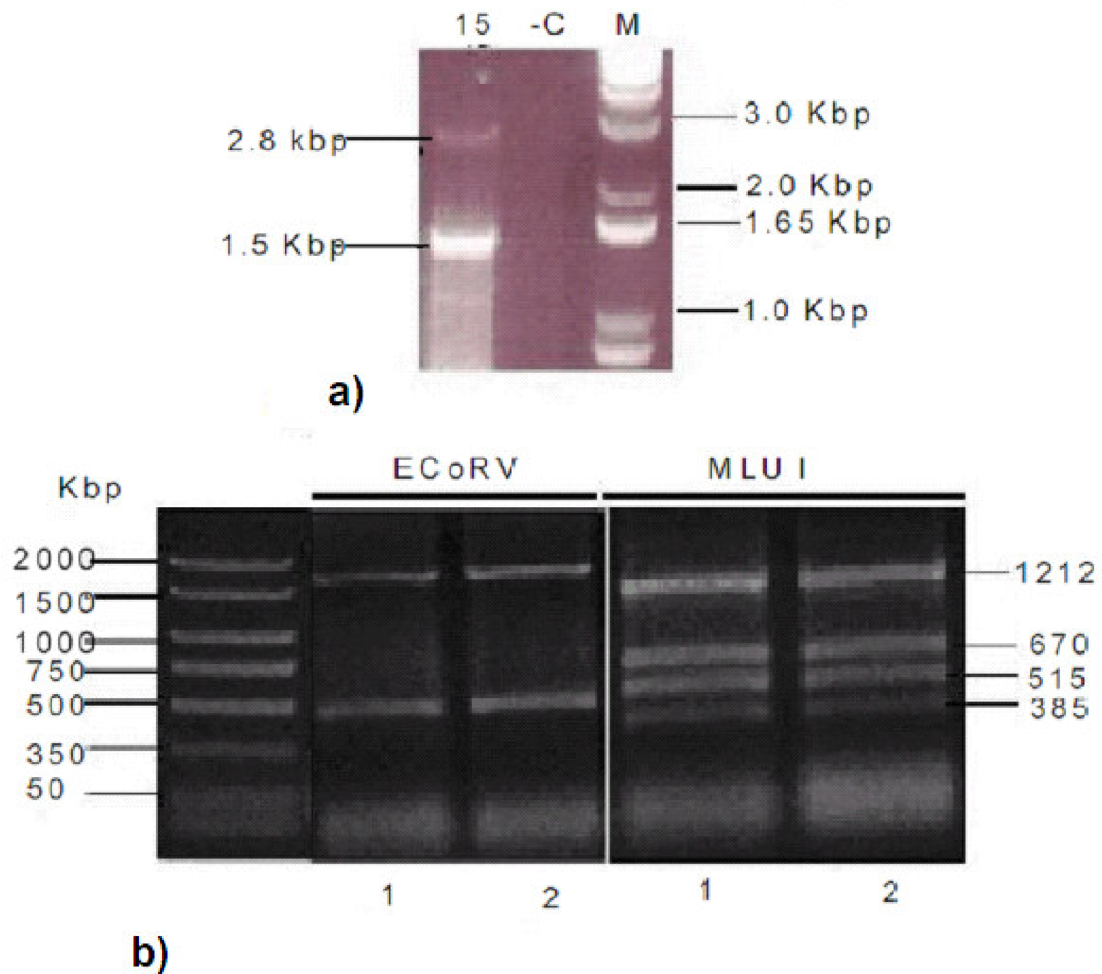

Fig. 4.2. Isolation and characterization of a circular 1.5 kbp subgenomic defective DNA molecule associated with CMG natural infection. (a) PCR-amplification of df DNA-A 15 from field sample number 15 and a near-full length 2.8 kbp of the helper virus (EACMV-TZ15) from the same plant. Lane –C was loaded with DNA extracted from a healthy cassava plant to serve as a negative control. (b) The characteristic banding patterns obtained after treatment of the PCR amplification products (2.8 kbp) of the helper virus with the restriction endonucleases *Eco*RV and *Mlu*I are indicated. DNA products were electrophoresed through an ethidium bromide-stained 1% agarose gel in 1% TAE buffer. Sizes of the DNA marker (M) are shown.

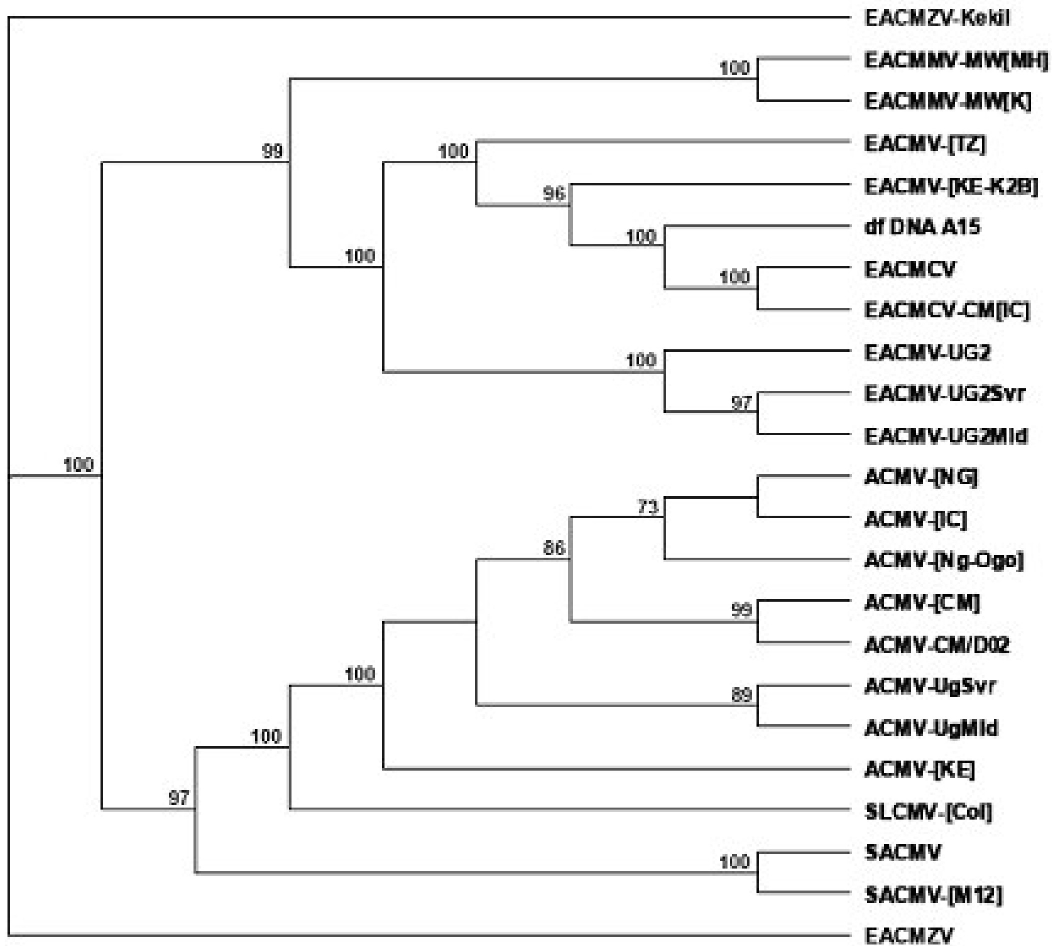

Fig. 4.3a. Phylogenetic tree obtained from the alignment of complete nucleotide sequences of DNA-A of 22 cassava mosaic geminiviruses with df DNA-A 15. The tree was generated using the Neighbor-joining method with the Mac Vector 7.2 (Accelry, San Diego, Inc. USA) computer program. The numbers at each branch indicate the percentage of 1000 bootstraps, which support the grouping at that node.

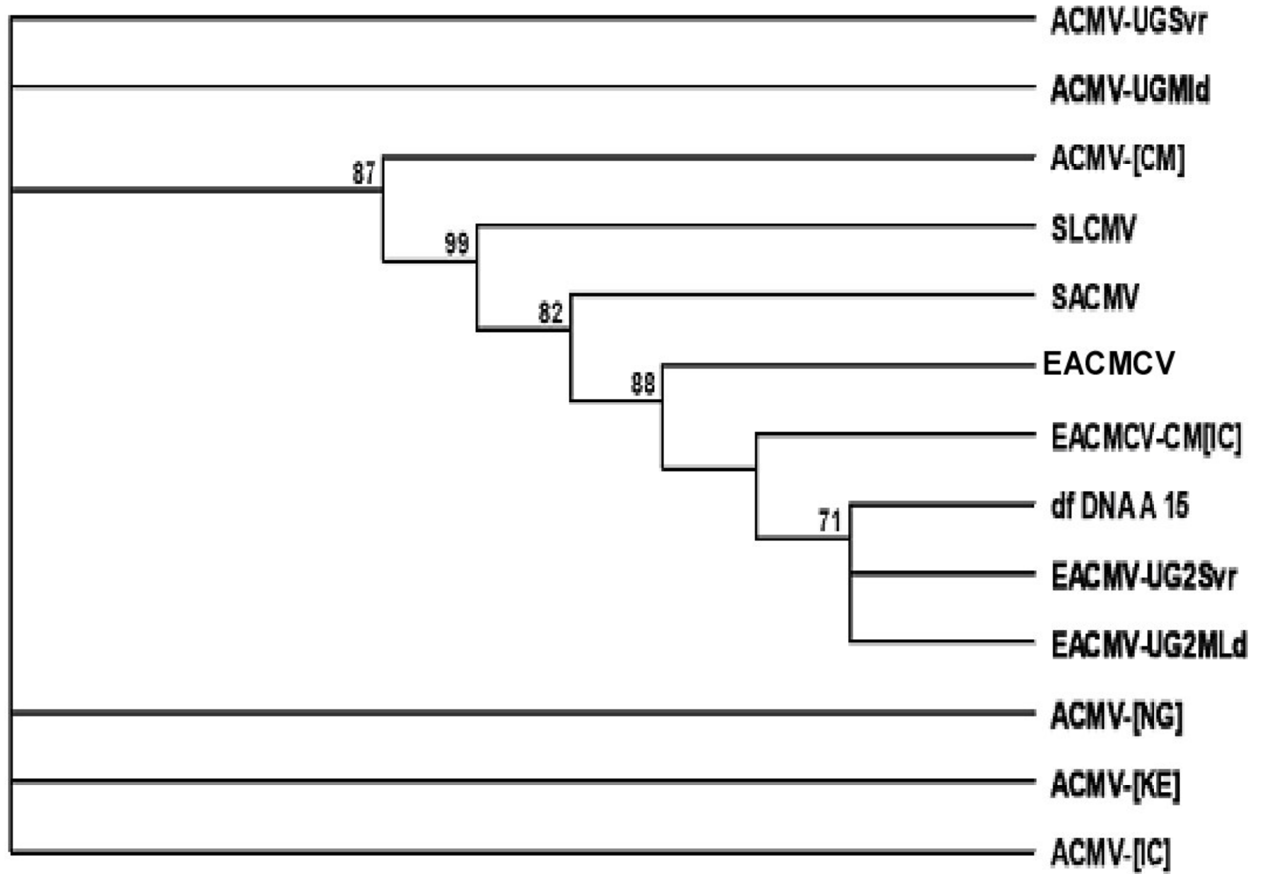

Fig. 4.3b. Consensus phylogenetic tree (1000 bootstrap replication) obtained from comparison of the CR sequences of selected cassava mosaic geminiviruses with the df DNA-A 15. Numbers indicate the percentage of bootstrap for each branch.

Fig. 4.4. Alignment of the nucleotide sequences of the CR of df DNA-A 15 with similar sequences of the EACMV isolates selected from GenBank. The iterated sequences (iterons), TATA box, the variable region and the invarint nanonucleotides (TAATATTAC) containing the origin of replication are indicated.

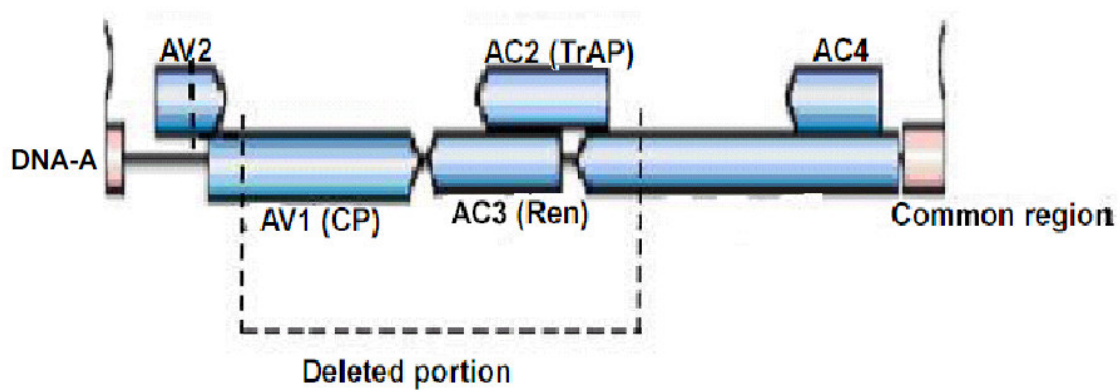

Fig. 4.5. Schematic genome organization of the subgenomic df DNA-A 15. Predicted ORFs in both orientations ('C' for complementary and 'V' for virion sense) are shown. The missing part of the genome based on the known genome of a full-length DNA-A component of EACMV is shown (dashed).

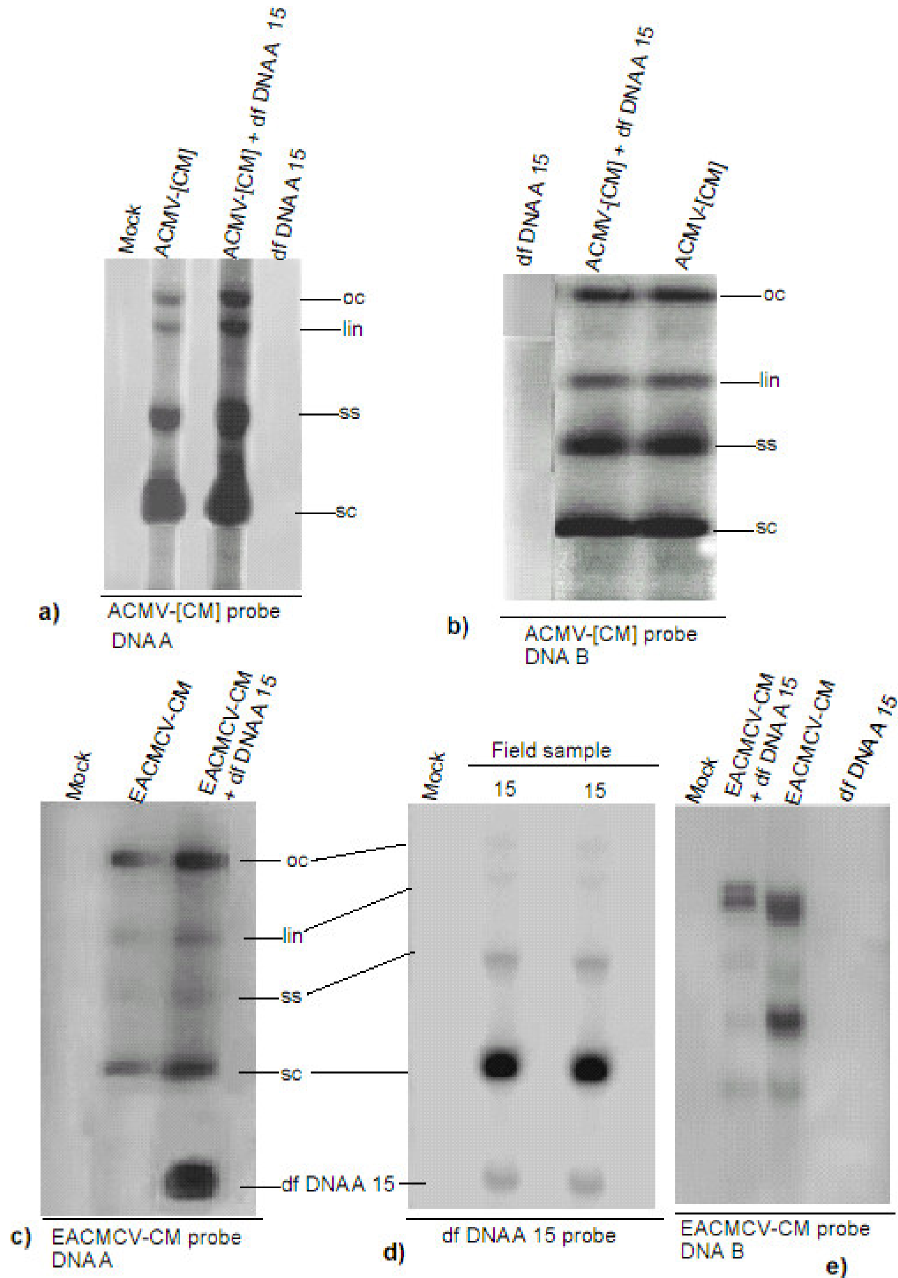

Fig. 4.6. Replication of df DNA-A 15 in *Nicotiana benthamiana* inoculated biolistically with clones of (a-b) ACMV-[CM] (DNA-A and -B), (c) EACMCV (DNA-A and -B). A blot of the DNA from original cassava obtained from the infected field plant is included in panel d in duplicate wells, (d) accumulation of EACMCV DNA-B inoculated singly or with df DNA-A 15. Positions of the replicative forms linear (Lin), open circular (OC), single stranded (SS), and super coiled (SC) are indicated.