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Identification of a defective molecule derived from DNA-A of the bipartite begomovirus of *East African cassava mosaic virus*

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Geminivirus defective interfering DNAs arise spontaneously in mechanically inoculated test plants, and have previously been found with DNA-B of the bipartite cassava mosaic geminiviruses, but not DNA-A. Reported here for the first time is the cloning and characterization of a naturally occurring truncated form of cassava mosaic geminivirus DNA-A, which at 1525 nt is around half the expected full size. Sequence analysis has shown it 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, and it has been termed df DNA-A 15. Phylogenetic comparisons placed the df DNA-A 15 molecule close to mild and severe isolates of EACMV-UG2. 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 with EACMCV singly inoculated plants, and there was an accumulation of df DNA-A 15 in systemically infected leaves. In addition, the level of EACMV DNA-B accumulation was reduced in the coinoculated plants compared with those inoculated with EACMCV alone. PCR and sequence analysis confirmed the helper virus as EACMV.

Keywords: cassava, cassava mosaic geminivirus, defective (df)DNA, DNA-A, EACMV, Tanzania

Introduction

Cassava mosaic disease (CMD) is widespread throughout Africa and is the most important constraint to cassava production. The disease is caused by cassava mosaic geminiviruses (CMGs) belonging to the genus *Begomovirus* of the family *Geminiviridae*. They are transmitted by the whitefly *Bemisia tabaci* and spread through infected cuttings, which is the usual mode of cassava propagation. *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) are the most commonly occurring CMGs (Swanson & Harrison, 1994). The CMG genome consists of two molecules of single-stranded DNA (DNA-A and DNA-B), each *c.* 2·8 kbp (Lazarowitz, 1992). DNA-A contains six partially overlapping open reading frames (ORFs) organized in two opposite transcriptional directions separated by an intergenic region (IR). On the virion-sense strand, DNA-A contains AV1 and AV2 ORFs, and AC1–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 IR (*c.* 200 nt) 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 molecules 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. Defective interfering (DI) molecules have been described for a few geminiviruses, and originate from sequence deletions of either DNA-A or -B. Choge *et al*. (2001) reported a DI of DNA-B of *South*

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African cassava mosaic virus that was isolated from the field-infected plant. However, no naturally occurring DI has been reported for DNA-A. They are normally about half the size of the full-length molecule of the geminivirus, 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). The genetic diversity and geographical distribution of CMGs occurring in Tanzania have been reported (Ndunguru *et al*., 2005). Here we report the results of a study undertaken to characterize a putative defective (df) DNA-A fragment PCR-amplified from a CMD-infected cassava plant from a cassava field in Tanzania.

Materials and methods

Source of viral DNA

Defective DNA was identified from a geminivirus-infected cassava plant collected in the Kagera region of north-western Tanzania in September 2002. A total of 90 young cassava leaf samples as well as cassava cuttings were collected. Cassava cuttings were planted in the growth chamber at the Donald Danforth Plant Science Center (DDPSC), St Louis, MO, USA and symptoms were monitored regularly on newly produced leaves for 60 days. Plants were kept at 28°C with a 16-h day length, and used as a source of DNA for subsequent analysis.

DNA extraction, PCR, cloning and sequencing

Subgenomic df DNA was amplified by PCR from a nucleic acid sample obtained from a field-collected CMD-infected cassava plant, and from a cutting of the same plant kept in a growth chamber at DDPSC. Total cellular DNA from cassava leaves of a plant collected in Tanzania and propagated in DDPSC was extracted by the method of Dellaporta *et al*. (1983). Initially, the universal primers UNI/F and UNI/R (Invitrogen), designed by Briddon & Markham (1994) to amplify near full-length DNA-A of CMGs (2·7–2·8 kbp), were used as described. In addition to the expected full-length (2·8-kbp) product, a 1·5-kbp PCR fragment was also amplified using the same primers. The PCR products were recovered after electrophoresis in a 1% agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen) following the manufacturer's instructions. The 2·8-kbp PCR products were first characterized by restriction analysis by digesting with *Eco*RV and *Mlu*I and then electrophoresed on a 1% agarose gel after staining with ethidium bromide. Both the 2·8- and 1·5-kbp PCR products were first ligated into pCR 2·1-TOPO using the TA Cloning Kit (Invitrogen) following the manufacturer's instructions, and cloned in *Escherichia coli* DH5α^{TM}-T1^{R}. Clones were confirmed for correct size inserts using *Eco*RI restriction digestion of the extracted plasmids, or PCR with primers used in the 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) was designed around an internal *Hin*dIII unique site (underlined) and used to amplify a 1·5-kbp PCR product from the original sample. The PCR products were purified as described above and cloned into pGEM-T Easy Vector (Promega) to produce pGEM df DNA-A 15.

Sequence analysis

Nucleotide sequences were assembled with the DNASTAR package. Multiple sequences were aligned using CLUSTAL-W of the MAC VECTOR 7.2 package (Accelry) to obtain nucleotide sequence identities using default parameters. The nucleotide sequences deleted from df DNA-A were ignored in the phylogenetic analysis using 'ignore gaps' option of CLUSTAL-W. The phylogenetic trees were constructed using a neighbour-joining majority rule consensus with 1000 bootstrapped replicates with MAC VECTOR 7.2. The CR sequences of DNA-A from CMGs were also assembled and analysed in a similar manner. Sequences for alignment were obtained from the GenBank database (Table 1). The MAPDRAW program of the DNASTAR package was used to analyse the ORFs in the df DNA-A 15. The extent of ORF disruption in df DNA-A 15 was identified by complete nucleotide sequence alignment with the closest related selected CMGs.

Construction of infectious clone

A partial dimer of the df DNA-A 15 was constructed as follows. A *Bam*HI–*Hin*dIII fragment of 645 bp from pGEM df DNA-A 15, containing the entire IR, was excised and cloned into *Bam*HI/*Hin*dIII double-digested pBluescript II SK+ (Stratagene), generating plasmid pSKdf DNA-A 15 (645). The full-length 1·5-kbp fragment was excised from pGEM df DNA-A 15 by digestion with *Hin*dIII and inserted into *Hin*dIII-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.

Infectivity assay

Infectivity of df DNA-A 15 was evaluated by biolistic inoculation of *Nicotiana benthamiana* with virus clones harbouring partial dimers of ACMV-Cameroon (ACMV-[CM]) and EACMV-Cameroon (EACMCV) essentially as described previously (Swanson & 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 inoculated biolistically with cloned DNA (100 ng per plant). The combinations were: (i) ACMV-[CM] (DNA-A and DNA-B); (ii) ACMV-[CM] (DNA-A and DNA-B) with df DNA-A 15; (iii) EACMCV (DNA-A and DNA-B); (iv) EACMCV (DNA-A

Table 1 Cassava mosaic geminiviruses (DNA-A) used in comparative sequence analysis and their respective nucleotide sequence databases

Name	Acronym	Accession number
African cassava mosaic virus-[Cameroon]	ACMV-[CM]	AF112352
African cassava mosaic virus-[Kenya]	ACMV-[KE]	J02057
African cassava mosaic virus-Uganda Mild	ACMV-UGMld	AF126800
African cassava mosaic virus-Uganda Severe	ACMV-UGSvr	AF126802
East African cassava mosaic Cameroon virus-Cameroon	EACMCV	AF112354
East African cassava mosaic Cameroon virus-Cameroon [Ivory Coast]	EACMCV-[CI]	AF259896
East African cassava mosaic virus-[Kenya-K2B]	EACMV-[KE-K2B]	AJ006458
East African cassava mosaic Malawi virus-Malawi [MH]	EACMMV-[MH]	AJ006459
East African cassava mosaic Malawi virus-Malawi [K]	EACMMV-[K]	AJ006460
East African cassava mosaic virus-[Tanzania]	EACMV-[TZ]	Z83256
East African cassava mosaic virus-Uganda2 (Uganda variant)	EACMV-UG2	Z83257
East African cassava mosaic virus-Uganda2 Mild	EACMV-UG2Mld	AF126804
East African cassava mosaic virus-Uganda2 Severe	EACMV-UG2Svr	AF126806
East African cassava mosaic Zanzibar virus	EACMZV	AF422174
East African cassava mosaic Zanzibar virus-[Kenya Kilifi]	EACMZV-[KE/Kil]	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

and DNA-B) with df DNA-A 15; (v) df DNA-A 15 alone. Noninoculated plants were used as controls. For each combination, five plants were inoculated. Inoculated plants were kept in a glasshouse, and disease symptoms were monitored visually for 35 days.

Southern blot analysis of replication

To compare accumulation of viral DNAs in the inoculated plants, total DNA was extracted from inoculated *N. benthamiana* 14 days postinoculation (dpi) essentially as previously described. Original field samples containing 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 insert in duplicate lanes. DNA replication was assessed by Southern blot analysis of the total DNA extract. A total of 5 μg was loaded on each well and separated on a 1% agarose gel by electrophoresis and transferred to Hybond-N+ nylon membranes (Amersham Pharmacia Biochem) and hybridized to probes specific to ACMV-[CM] or EACMCV genomic components (DNA-A and B) (Fondong *et al*., 2000). Probes were labelled 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 the full-length 1·5-kbp DNA *Hin*dIII fragment of pGEM df DNA-A 15.

Results

Symptoms on cassava plants

In the field, the cassava plant containing the df DNA-A 15 expressed moderate mosaic, leaf distortion and yellowing (Fig. 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 (Fig. 1b).

Polymerase chain reaction

PCR confirmed that df DNA-A 15 was present in the cassava leaf sample collected from a CMD-infected cassava field (Fig. 2a). Only one sample from a single field out of the 90 samples collected from different cassava-growing areas in the Kagera region produced this subgenomic fragment. Primer pair UNI/F and UNI/R also amplified the 2·8 kbp expected for the near full-length DNA-A component of the CMG. PCR of leaf extracts from the same plant grown in the growth chamber yielded a 1·5-kbp PCR product 1 and 5 months after planting, using both primer pairs UNI/F/R and DI 15H/F/R.

Identification of 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. 2b). Comparison of the partial nucleotide sequence of the 593 nt beginning at the UNI/F primer site in the gene coding for AC1 protein of 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 of 96, 98, 97 and 93%, respectively.

Phylogenetic comparisons of df DNA-A 15 with selected begomoviruses

Begomoviruses used in comparative sequence analyses, their respective acronyms and GenBank accession numbers are shown in Table 1. The complete nucleotide sequence of the df DNA-A 15 is available in the GenBank database (accession number AY676464), together with its helper virus EACMV-[TZ15] (AY828226). The df DNA-A 15 clone was 1525 nt long, some 55% of the DNA-A

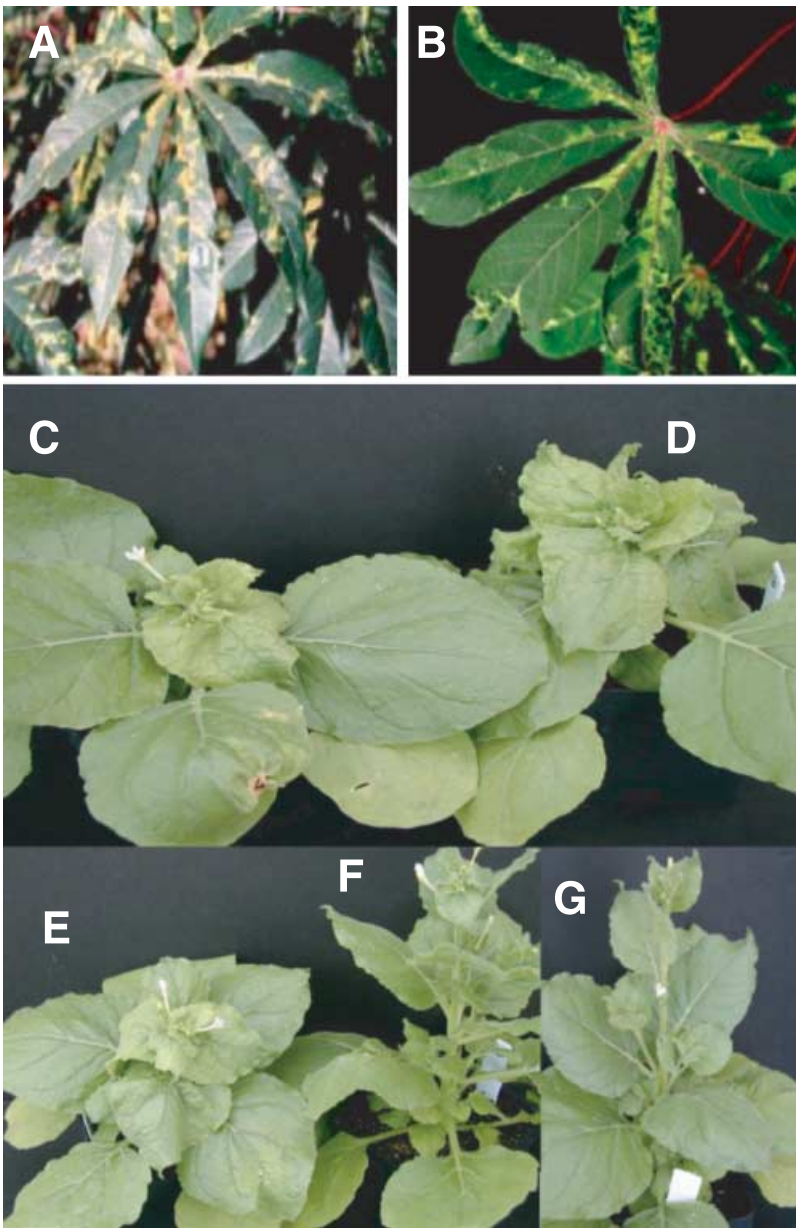

Figure 1 Disease symptoms of cassava mosaic geminiviruses (CMGs): (a) on field-grown cassava plant containing df DNA-A 15; (b) reproduced on cassava in the growth chamber. *Nicotiana benthamiana plants* 14 days after inoculation with (c) ACMV-[CM]; (d) ACMV-[CM] + df DNA-A15; (e) EACMCV; (f) EACMCV + df DNA-A 15; (g) df DNA-A 15.

genome of other CMGs. The phylogenetic analysis based on alignments of the corresponding nucleotide sequences of DNA-A components of CMGs is shown in Fig. 3. The df DNA-A 15 clearly groups with EACMV species with the highest overall nucleotide sequence identity found with EACMV-UG2Svr (96%), a low identity with SLCMV-[Col] (52%) and low (< 50%) sequence homology to the ACMV isolates.

As high levels of nucleotide sequence identity between the two geminivirus isolates constitute strong evidence that they are closely related, a comparison of the nucleotide sequence of df DNA-A 15 CR was made with corresponding published CR sequences of begomoviruses. The comparison revealed df DNA-A 15 to have high sequence similarity to both EACMV-UG2Svr (97%) and EACMV-UG2Mld (96%), and all three clustered closely in the phylogenetic analysis (Fig. 4). It had nucleotide sequence identity of 80% to EACMCV, and differed greatly from the ACMV isolates with < 50% sequence identity.

To examine more closely the nature of regulatory sequences of the df DNA-A 15, the 185-nt CR was aligned with four EACMV isolates. This comparison revealed the typical motifs conserved in cassava begomovirus CR sequences and, in all significant respects, was identical to EACMV's.

Analysis of df DNA-A 15 ORFs

The complete sequences of two DNA-A components of EACMCV and EACMV-UG2Svr were used for size

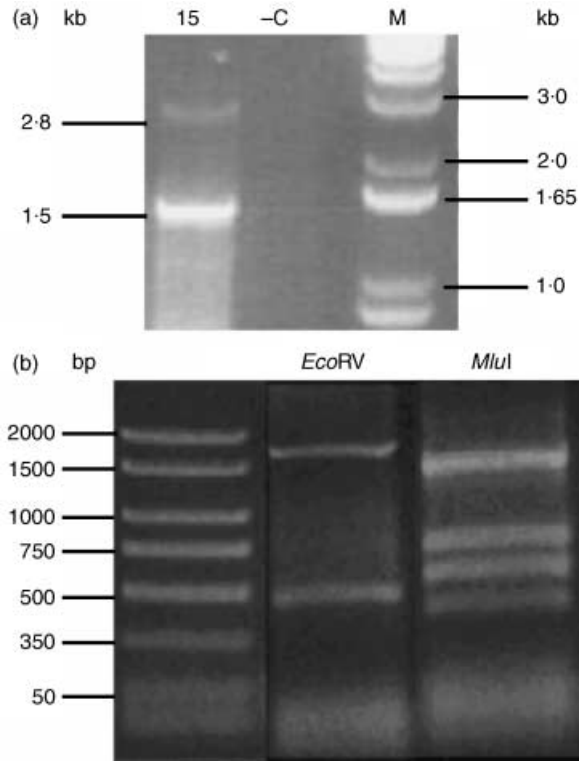

Figure 2 Isolation and characterization of DNA-A molecules associated with cassava mosaic geminiviruses (CMGs) in natural infection. (a) PCR amplification using primers UNI/F and UNI/R of DNA extracted from field-grown cassava plant (15) and a symptomless (–C) cassava plant. (b) Characteristic banding patterns obtained after treatment of the 2·8 kb PCR amplification products from (a) with restriction endonucleases *Eco*RV and *Mlu*I. DNA products were electrophoresed through an ethidium bromide-stained 1% agarose gel in 1% TAE buffer. Sizes of co-electrophoresed DNA marker bands (lane M) are shown.

comparison with the ORFs of df DNA-A 15. On the virion-sense strand, two ORFs were identified, AV2 and AV1, 109 and 56 amino acids (AAs) long, respectively. The C-terminal sequence of AV2 and a large portion of AV1 had been deleted. In the complementary-sense strand, only two ORFs were found. *AC4* was the only intact gene at 78 aa long. The *Rep* gene was 290 aa long with part of its C-terminal sequence deleted. Two ORFs, AC2 and AC3, always found in the DNA-A component of other CMGs, were missing in df DNA-A 15 (Fig. 5).

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

Nicotiana benthamiana plants coinoculated biolistically with ACMV-[CM] and df DNA-A 15 developed systemic mosaic symptoms at 5 dpi that were indistinguishable from those induced by ACMV-[CM] alone (Fig. 1c,d), and plants recovered equally at *c.* 21 dpi. Plants inoculated with EACMCV alone expressed severe systemic symptoms starting at 6 dpi, displaying mosaic and downward leaf curling, but did not recover (Fig. 1e). In contrast, all the plants coinoculated with EACMCV and df DNA-A 15 developed only mild systemic mosaic symptoms, with very slight leaf distortion at 13 dpi (Fig. 1f). Plants inoculated with df DNA-A 15 alone remained symptomless throughout the experiments (Fig. 1g).

Trans-replication and maintenance of df DNA-A 15

Southern blot analysis of total DNA collected from systemically affected leaves of *N. benthamiana* inoculated with the ACMV-[CM] and df DNA-A 15 combination revealed no significant change in the levels of viral DNA of both components DNA-A and DNA-B (Fig. 6a,b). The df DNA-A 15 was detected in systemically infected tissue after coinoculation with EACMCV, but not when df DNA-A 15 was inoculated alone (Fig. 6c). Analysis of the viral DNA showed a reduced level of EACMCV DNA-B accumulation in plants inoculated with both EACMCV and df DNA-A 15 (Fig. 6d). The df DNA-A 15 replication form was also detected in the original field sample, although in a smaller amount compared with that observed in *N. benthamiana* (Fig. 6e).

Discussion

This study has demonstrated for the first time the occurrence in nature of a df DNA-A of EACMCV in cassava. Choge *et al.* (2001) reported the occurrence of DI of DNA-B of SACMV in the field in South Africa, and DI of DNA-B has been reported in *N. benthamiana* (Stanley & Townsend, 1985). The df DNA-A 15 was detected in a CMD-infected cassava plant in the field, and investigation of the sequence confirmed it to be a truncated EACMV DNA-A. The sequence confirmed the presence of IR and the 5′ part of the *AV1* gene and *c.* 80% of the *AC1* gene. Subgenomic df DNA molecules, associated with a number of begomoviruses (Stanley & Townsend, 1985; MacDowell *et al.*, 1986; Stanley *et al.*, 1997), seem to be fairly uniform in structure and all retain their IR and a large portion (5′ end) of *AC1* ORFs, as observed in this study. The IR contains the origin of replication, without which the molecule cannot be maintained (Stanley & Townsend, 1985).

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 the 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 CR of the df DNA-A 15 was compared to other EACMV isolates. A partial sequence of EACMV-[TZ15], the helper virus of df DNA-A 15, showed high nucleotide sequence identity to the df molecule and other EACMV isolates, suggesting that df DNA-A 15 is indeed derived from EACMV.

Defective interfering molecules are also associated with many plant- and nearly all animal-RNA viruses (Simon

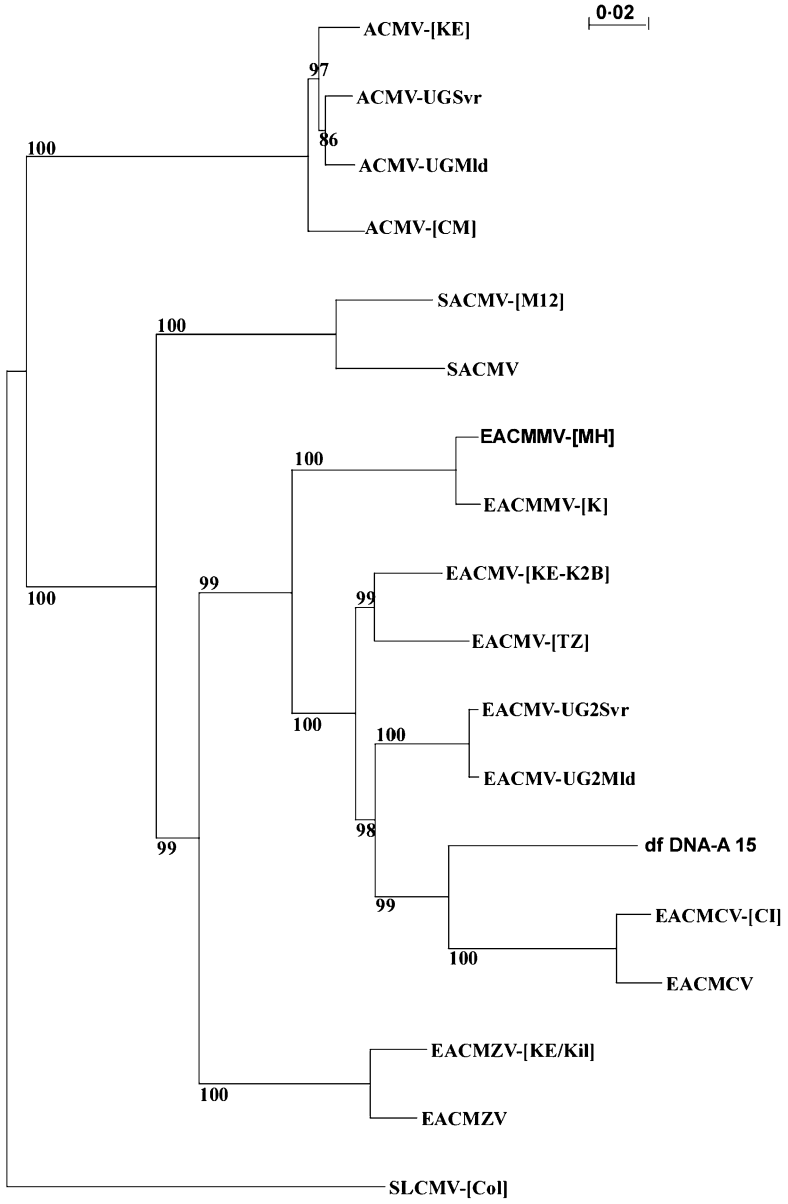

Figure 3 Phylogenetic tree obtained from alignment of the nucleotide sequences of DNA-A components of cassava mosaic geminiviruses with corresponding sequence of df DNA-A 15 (1000 bootstrap replications). Sequence of Sri Lankan cassava mosaic virus-[Colombo] used as an outgroup.

et al., 2004), and contain all the *cis*-acting elements necessary for RNA-dependent RNA polymerases of the parental virus. Defective molecules are also described for the leafhopper transmitted geminivirus *Beet curly top virus* (BCTV), a Curtovirus (Stenger *et al*., 1992), the genome of which contains elements similar to those in begomovirus DNA-A. Moreover, the BCTV df molecules resemble those associated with cotton leaf curl disease in retaining the IR 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* gene, as observed earlier (Liu *et al*., 1998). This molecule could then be replicated in substantial amounts by the normal mechanism used by all geminiviruses.

The biological effect of df DNA-A 15 was determined experimentally in the glasshouse using biolistic inoculation of *N. benthamiana*. Defective interfering DNA spontaneously produced by geminiviruses reduces 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 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 *Rep* gene-binding motif for df DNA-A 15 is GGTGGAATGGGGG, identical to that for EACMCV. The df DNA-A 15 DNA-A 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

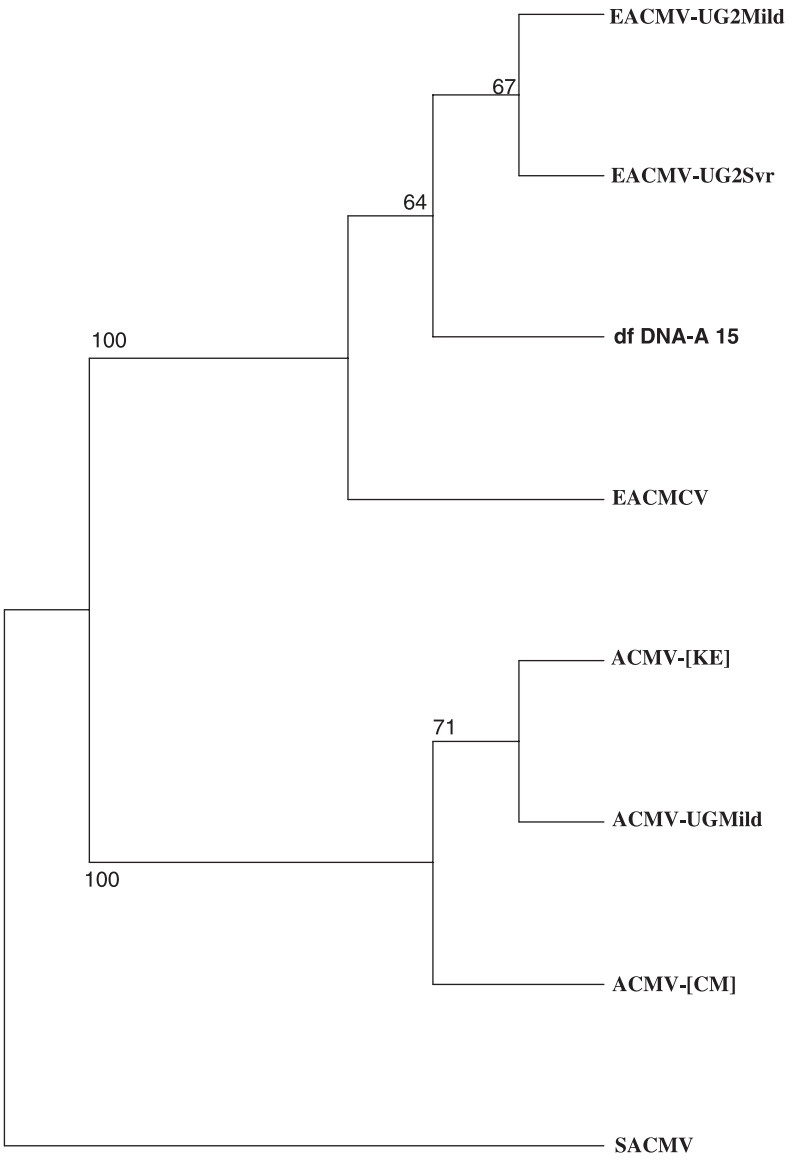

Figure 4 Consensus phylogenetic tree (1000 bootstrap replication) obtained from comparison of common region sequences of selected cassava mosaic geminiviruses with df DNA-A 15. *South African cassava mosaic virus* was used as an outgroup.

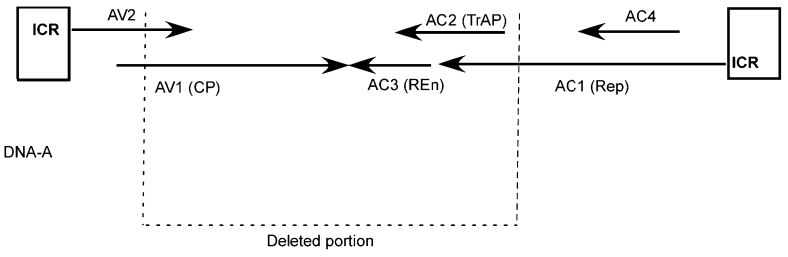

Figure 5 Schematic genome organization of subgenomic df DNA-A 15. Predicted ORFs in both orientations: ACx for complementary sense; AVx for virion sense. The deleted part of the genome based on the known genome of a full-length DNA-A component of EACMV is shown (dashed).

Rep gene. ACMV-[CM] contains a *Rep* gene-binding motif repeat of TGGAGACA (Fondong *et al*., 2000), different from that found in df DNA-A 15. Thus *trans*-replication is very unlikely because of probable sequence incompatibility, and there is no alteration in the pattern or concentration of helper virus DNAs which are normally seen in infections that contain defective viral components. Plants coinoculated with ACMV-[CM] and df DNA-A 15 produced systemic symptoms indistinguishable from that induced by ACMV-[CM] alone. Although a *trans*-encapsidation study was not carried out, the size of the df DNA-A 15, and the fact that it was found in only a single plant, might suggest it is not encapsidated.

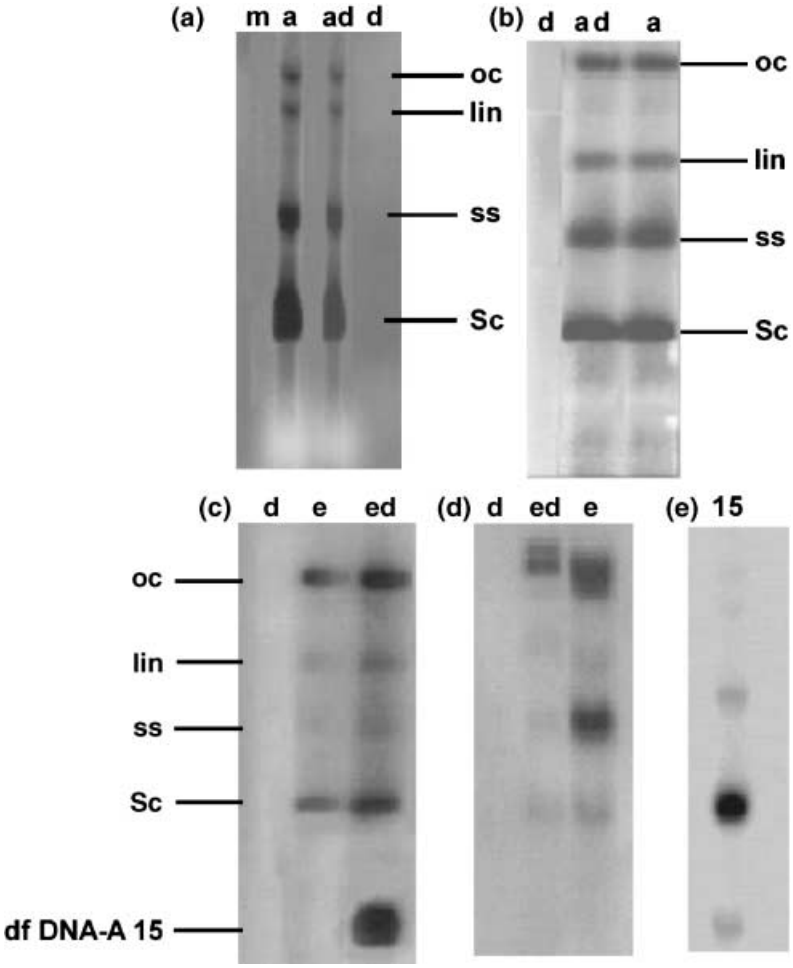

Figure 6 *Trans*-replication and maintenance of df DNA-A 15 under artificial and natural infection conditions. *Nicotiana benthamiana* was inoculated biolistically as follows: **a**, ACMV-[CM]; **d**, df DNA-A 15; **ad**, ACMV-[CM] and df DNA-A 15; **e**, EACMCV; **ed**, EACMCV and df DNA-A 15; **m**, mock. An equal amount (5 μg) of DNA isolated 14 dpi was electrophoretically separated and the gel blotted. The blot was probed with: (a) ACMV-[CM] DNA-A; (b) ACMV-[CM] DNA-B; (c) EACMCV DNA-A; (d) EACMCV-DNA-B. (e) DNA isolated from field-grown cassava plant 15, probed with df DNA-A 15. Positions of replicative forms linear (**lin**), open circular (**oc**), single stranded (**ss**) and supercoiled (**Sc**) are indicated.

It has been suggested that defective genomes gain a replication advantage over the wild type simply because they are shorter. In addition, selection favours 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 accumulated to only a low level. As DNA-B carries the *BC1* gene that encodes for symptom expression, low levels of DNA-B may contribute to symptom attenuation. This was consistent with the mild symptoms observed in all the plants inoculated with the EACMCV and df DNA-A 15 combination. The possibility of the truncated *Rep* gene of df DNA-A 15 acting as a dominant negative mutant rather than as a DI molecule, together with *trans*-encapsidation and transmission, remain to be investigated to gain an understanding of the mechanism of symptom modulation by DIs.

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