CHAPTER 5

Two novel satellite DNA molecules associated with bipartite cassava mosaic begomoviruses enhance symptoms and break resistance in a cassava germplasm

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We report here the discovery of two satellite ssDNA molecules associated with natural cassava mosaic geminivirus infections in Africa. These molecules, designated satDNA-II and satDNA-III, are dependent on geminiviruses for replication and movement within the

host. With sizes of 1.0 and 1.2 kbp respectively, they are distinct from each other (23% nts identity) and from all other sequences, but they possess putative *cis*-acting elements, suggesting their ability to code for functional proteins. When present in coinfections with geminiviruses, the satellites caused novel, severe disease symptoms and increased viral accumulation. In addition, high resistance to geminivirus infection in the landrace TME3, an important component of cassava improvement programs, can be broken by satDNA-II and satDNA-III in a single cycle of infection. Concern is raised regarding the impact of these satellites on food production and their putative role in the current pandemic of cassava mosaic disease.

5.1 INTRODUCTION

Plant viruses are often associated with satellite molecules which are able to modulate replication and symptom expression of their helper viruses (Roossinck *et al*., 1992). Satellites are best characterized in infectious RNA systems (Francki, 1985) where they are dependent on the helper virus for replication, encapsidation, and dissemination. Geminiviruses (family *Geminiviridae*) are plant viruses with one (monopartite) or two (bipartite) circular, single stranded DNA (ssDNA) genomes that replicate in the nuclei of infected plant cells via a double stranded DNA (dsDNA) replicative form (Hanley-Bowdoin *et al*., 1999). Recently, novel, circular, single-stranded satellite DNAs (satDNAs), approximately half the size of their helper virus, have been found associated with monopartite geminiviruses. These include a satDNA molecule of about 682 nucleotides (nts) associated *with tomato leaf curl virus* (ToLCV) in Australia (Dry *et al*., 1997) named DNA-1, and DNA-β molecules of 1367 nts and 1347 nts respectively, found associated with, *cotton leaf curl Multan virus* (CLCuMV) (Mansoor *et al*., 1999; Briddon *et al*., 2004), and *ageratum yellow vein virus* (AYVV) (Briddon *et al*., 2000; Saunders *et al*., 2000). Since their discovery, numerous sequences of these types of satellites have been discovered in the Old World exclusively associated with different species of monopartite geminiviruses (Bull *et al*., 2003; Zhou *et al*., 2003). DNA-β has been shown to play a direct role in symptom induction, impacting host range determination and

facilitating accumulation of the begomoviruses and their encoded pathogenicity factors (Saunders *et al*., 2002; Mansoor *et al*., 2003; Stanley, 2004). These satellites are co-encapsidated and whitefly-transmitted with their respective helper geminivuses. To date, only monopartite begomoviruses are reported to be associated with satellite DNA-1 and DNA-β (Bull *et al*., 2003; Mansoor *et al*., 2003). For genome organization of these satellites and a review of current knowledge see Mansoor *et al*. (2003).

Cassava mosaic geminiviruses (CMGs) belong to the genus *Begomovirus* and are transmitted by the whitefly *Bemisia tabaci* and through planting of infected cassava cuttings. The CMGs genome is divided into two components, designated DNA-A and DNA-B (Stanley, 1983; Stanley *et al*., 2004) both of which are required for virus proliferation. The presence of both components in infected plants is sufficient to produce typical disease symptoms. Cassava can be infected by CMGs belonging to 6 different species in Africa (*African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), and *South African cassava mosaic virus* (SACMV), and 2 species in the Indian sub-continent (*Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV))(Fauquet and Stanley, 2003). Symptom variability in infected cassava plants, which ranges from mild to very severe, has been attributed to variation in virus strains, host susceptibility, age at which virus infection is established and extend of vector activity (Thresh *et al*., 1998). Host susceptibility is variable with some germplasm like the cassava landrace TME3 considered extremely resistant to CMGs. Using classical genetic analysis and molecular mapping, Akano *et al*. (2002) demonstrated that a major dominant gene controls resistance to CMGS in TME3. This landrace has proven to be highly resistant, but not immune, to infection with geminiviruses in the field and to *East African mosaic virus* Ugandan strain (EACMV-UG2), *African cassava mosaic virus* Cameroon isolate (ACMV-[CM]), *East African cassava mosaic* virus Cameroon isolate (EACMV-[CM]) and *Sri Lankan cassava mosaic virus* (SLCMV) by biolistic inoculation in the laboratory (Fofana *et al*., unpublished results).

In a recent survey of cassava mosaic disease (CMD) in Tanzania, unique symptoms were found associated with some virus isolates (Ndunguru, submitted). Agarose gel fractionation of total DNA extracts from some of these leaf samples showed the presence of subgenomic-size components, which could not be amplified by PCR using CMG, specific, or universal degenerate primers. This observation prompted us to investigate the possible involvement of additional molecules associated with CMD in these plants.

Here we describe the discovery of two previously unreported satDNA molecules associated with CMGs in cassava. This represents the first report of satDNAs to be found associated with bipartite begomoviruses. The impact of this observation in the understanding of the aetiology, nature and complexity of CMD is discussed.

5.2 MATERIALS AND METHODS

5.2.1 Virus sources and DNA extraction. In September 2002, leaf samples were collected from naturally infected cassava plants showing cassava mosaic disease symptoms from northwestern, southern, and eastern coast of Tanzania. Selected cassava cuttings 15-20 cm in length were also taken from these symptomatic plants, transported to the DDPSC, St Louis, Mo, USA, planted in soiless compost in 10 cm pots and placed in a growth chamber. Stakes were grown at 28^{o}C with a 16 hour day length. Young, newly unfolded leaves showing CMD symptoms were taken from field and growth chamber grown plants and total DNA extracted as described by Dellaporta *et al*. (1983).

5.2.2 Polymerase chain reaction for amplification of viral DNA. A universal primer pair Beta01(5′-GGTACCACTACGCAGCAGCC-3′) and Beta02 (5′-GGTACCTACCCTCCCAGGGGTACAC-3′) (Briddon *et al*., 2001) were used to amplify possible full-length satDNA-II from cassava mosaic disease-infected plant DNA extracts. The K*pn*I restriction endonuclease site (underlined) was introduced in both primers to facilitate cloning. For amplification of the possible full-length satDNA-III, primers DNA-1/F (5′-TGGGGATCCTAGGATATAAATAACACGTC-3′) and DNA-1/R (5′-

CTAGGATCCGGACAAATTACAAGCGTA-3′) with *Bam*HI unique site (underlined) (Mansoor *et al*., 1999) were used. PCR with 35 cycles each consisting of 1 min at 94°C, 1 min at 59°C, and 2 min at 72°C was performed for satDNA-III and a lower annealing temperature of 50°C for amplification of satDNA-II.

5.2.3 Cloning and sequencing. PCR products were cloned into pGEM®-T Easy Vector (Promega, USA) as described by the manufacturer and the ligation mix transformed into competent *Escherichia coli* strain JM109 (Promega, USA). After an overnight multiplication at 37°C, plasmids were purified by Qiagen DNA purification kit. SatDNA-II and satDNA-III DNA sequences were determined in both orientations by automated sequencing at the Protein and Nucleic Acid Chemistry Laboratories (PNACL), Washington University School of Medicine, ST. Louis Mo, USA (ABI377 DNA sequencer, Perkin Elmer, Foster City, CA).

5.2.4.Genome sequence analysis. Sequence data were assembled with the aid of DNAStar software. FramePlot 2.3.2 software (Ishikawa and Hott, 1999) was used for analysis of open reading frames (ORFs). CpGplot (EMBL-EBI, European Bioinformatic Institute) was employed to scan for the presence of regions with high GC content in the complete nucleotide sequence and TRANSFAC® 6.0 –Public (**http://www.gene-regulation.com/pub/programs/alibaba2/index.html**). Multiple alignments of the GC-rich region nucleotide sequences was performed using Jellyfish Version 3.0 (Lavelocity, Inc, USA) computer program using default parameters.

5.2.5 Construction of infectious clones. The plasmid vector pGEM-T Easy harboring satDNA-II (pGEM-satDNA-II) was digested with *Kpn*I to release a full-length satDNA-II. Both the resulting satDNA-II fragment and the linearised plasmid DNA were recovered from the agarose gel and purified. The satDNA-II with *Kpn*I on both ends was re-ligated back to the *Kpn*I-linearised pGEM-T Easy vector plasmid assuming that in the ligation reaction, some inserts would first form dimers before ligation to the vector.

Confirmation of dimer formation was done by digestion with *EcoR*I restriction endonuclease and correct orientation checked by digestion with appropriate restriction enzymes chosen from the internal site of satDNA-II sequence. A dimer of satDNA-III (pGEM-2satDNA-III) was generated using the same strategy described for satDNA-II except that satDNA-III from clone pGEMT-satDNA-III was released by digestion with *Bam*HI.

5.2.6 Investigation of a possible biological role of satDNA-II and -III. To observe the effect of satDNA II and satDNA III on infectivity of *Nicotiana benthamiana*, the highly CMD-resistant cassava landrace TME3 and the susceptible cultivar TMS 60444, plasmids bearing tandem repeated copies of the cloned inserts (pGEM-2satDNAII and pGEM-2satDNAIII) were delivered to test plants by biolistic inoculation together with infectious clones harboring partial dimers of ACMV-[CM], EACMV-UG2 and EACMCV-CM (DNA-A and -B components) as described earlier (Pita *et al*., 2001).

5.2.7 Southern blot analysis. Levels of DNA replication for satDNA-II, satDNA-III and the helper geminiviruses were determined by Southern blot analysis of total cellular DNA extracted from biolistically inoculated *N. benthamiana* two weeks after inoculation and from cassava 21 dpi using the procedure of Dellaporta *et al*. (1983). The Southern blot procedure was as described earlier (Fondong *et al*., 2000; Pita *et al*., 2001).

5.3 RESULTS

5.3.1 Disease symptoms in cassava plants containing satellite molecules

In the field, plants subsequently found to contain satDNA-II generally displayed very severe symptoms, characterized by leaf distortion, yellowing and mosaic (Fig. 5.1b, 1c).

Cassava plants in which satDNA-III was detected expressed very unique symptoms, characterized by a severe leaf narrowing (filiform) due to loss of leaf lamina and at times very prominent leaf yellowing (Fig. 5.1c). Similar symptoms were reproduced from cuttings from the same plants grown in a growth chamber.

5.3.2 Isolation of satDNA-II and satDNA-III

Leaf samples expressing unique symptoms of CMD were collected in Tanzania within in the major cassava growing areas (south, coastal area, and Lake Victoria basin). Woody stem cuttings were also collected and planted in a growth chamber at the DDPSC for symptom reproduction. Electrophoretic separation of DNA extracts from plants expressing unique symptom characteristics revealed the presence of two distinct DNA bands smaller than the expected full-length (2.7-2.8 kbp) genome of CMGs. Universal primers designed for amplification of full-length cassava begomoviruses could not amplify any DNA from these subgenomic-size bands. Different primer combinations were designed based on published sequences of satellite DNA molecules to amplify the subgenomic-size DNA. Using universal primers Beta01 and Beta02 designed within the conserved sequence of DNA- β (Briddon *et al*., 2001), a PCR product of 1.0 kbp (Fig. 5.2a) was obtained (designated here as satDNA-II) in 25 samples out of 187, collected from the coastal area in the Pwani district and the Lake Victoria basin (northwestern Tanzania). Using primers DNA-1/F and DNA-1/R (Mansoor *et al*., 1999), a 1.2 kbp PCR product (Fig. 5.2b)(designated here as satDNA-III) was amplified in 35 samples out of 65 from the Lake Victoria basin (approximately 1200 km from the coastal area). In all the above PCR reactions using these primers, DNA from healthy control cassava plants produced no products (Fig. 5.2a, 2b).

5.3.3 Sequence analysis

To confirm that recovered sequences of satDNA-II and satDNA-III originated from a circular DNA and represent full-length copies, PCR products amplified from individual infected plant samples were cloned and sequenced. Data confirmed that termini of the satDNA-II and satDNA-III inserts were contiguous in the amplified DNA fragments, thereby verifying the circular nature of the DNA. The complete nucleotide sequence of satDNA-II and satDNA-III molecules were determined to be 1032 bp and 1209 bp in length respectively (Fig. 5.3a,b). The nucleotide and amino acid sequences derived from these sequences were blasted against GenBank sequences but produced no significant match against any known geminiviruses. This strongly indicated that these molecules were not defective DNAs from geminiviruses nor were they PCR artifacts, since DNA from healthy cassava plants gave no amplification. Extensive searches of the non-redundant nucleotide database with satDNA-II and satDNA-III sequences also failed to reveal significant matches with any existing sequences including previous reported DNA satellites (Dry *et al*., 1997; Mansoor *et al*., 1999; Briddon *et al*., 2000). However, it is important to note that these search comparisons detected several short sequences varying from 13 to 33 nts, that have high levels of identity with widely conserved sequences in many different types of organisms including animals, plants, bacteria, fungi, algae and even vertebrate viruses. The significance of these short stretches of common sequences remains unknown. Sequences obtained from the PCR products revealed that only forward primers (Beta01 for satDNA-II and DNA-1/F for satDNA-III) were involved in the PCR amplification by annealing in the opposite orientation at both ends of the sequence. Sequence inspection shows the presence of inverted terminal repeats at the end of each satDNA sequence capable of forming a panhandle-like structure when folded. This situation is similar to that obtained for adeno-associated viruses for which the origin of replication is mapped and replication initiated by self-priming (Muzyczka and Berns, 2001).

5.3.4 Structure features of satDNA-II

DNA sequence comparison clearly indicated satDNA-II and satDNA-III to be distinct molecules with only 23% sequence homology. Neither satellite was found to contain the conserved nanonucleotide sequence TAATATTAC found in geminiviruses and in DNA-β satellites, nor the TAGTATTAC sequence found in DNA-1 to which the origin of replication has been mapped (Hanley-Bowdoin *et al*., 1999; Mansoor *et al*., 2003). Using CpGplot software, a region of high (66.7%) CG composition (239 nts long) was identified between nts 49-287 (Fig. 5.3a) (numbering is according to the satDNA-II insert), which is characterized by the presence of direct repeats of short hexanuclotides CCGCCG (repeated five times), pentanucloetides CCGCC (repeated twice), as well as trinucloetides CGC (Fig. 5.3a). Analysis further revealed the presence of one putative TATA box binding protein (TBP) motif (TATAAAT) at nts 913-919 and a putative consensus transcription polyadenylation signal AATAAA (Fig. 5.3a). Interestingly, a TTGTA motif frequently associated with functional polyadenylation signals (Rothnie *et al*., 1994; Sanfacon, 1994), is positioned upstream of the polyadenylation signal. SatDNA-II is predicted to have three putative activator protein-1 (AP-1) binding motifs (TGCA) (Fig. 5.3b) whose roles are yet to be determined, although such a motif has been implicated in activation of gene expression in animal systems (Kawasaki *et al*., 2001). It is not clear if the presence of any of these motifs indicates association with a plausible promoter that is expressed, although these observations suggest that satDNA-II is a transcriptionally active molecule. A similar motif is found in CMG upstream or downstream of the polyadenylation signal. A sequence search for Rep-binding element (GGTGGAATGGGGG) in satDNA-II, similar to that found in EACMV isolates (Fondong *et al*., 2000; Pita *et al*., 2001) and ACMV isolates (GGAGACA) (Fondong *et al*., 2000) was unsuccessful. Sequence analysis identified a total of eight ORFs (Fig. 5.4b) three and five on the sense (V) and complementary (C) strands, respectively. ORF V1 is the longest (52 aa) and C4 the shortest (24 aa).

5.3.5 Structure features of satDNA-III

The sequence of satDNA III is presented in Fig. 5.3b. This satellite contains a region of high CG-content (nts 110-338) characterized by perfect short repeats (tri, tetra, penta and hexa-nts). The pentanucleotide CCGCC for example is repeated six times in the genome. GC-rich region nucleotide sequences of satDNA-II and satDNA-III however, showed 51% identity. Furthermore, it has three putative TATA binding protein (TBP) sites, nts 13-22 (GATATAAATA), nts 932-943 (TACATATATAT), and nts 1150-1159 (TCTGTATATA) (Fig. 5.3b). One consensus transcript polyadenylation signal (AATAAA) (nts 576-580) is present and is preceded with a stop codon TGA. A TTGTA motif (nts 551-555) is positioned upstream of the polyadenylation signal. Upstream of the TTGTA motif, there is a hexamer sequence, ACGTCA which also occurs in several ssDNA plant viruses believed to be associated with strong promoter activity in undifferentiated actively dividing cell types (Nakayama *et al*., 1992). This motif is also a part of a predicted putative cAMP-responsive element (CRE) binding site CTACGTCA responsible for activation of eukaryotic gene transcription (Louet *et al*., 2002). SatDNA-III contains a poly-A sequence (nts 40-56), immediately followed by a direct repeats tri-nts (TAATAATAA) whose role is yet to be established, although this may be a result of sequence duplication to satisfy size requirements for encapsidation and or virus movement as discussed below. Like satDNA-II, satDNA-III does not contain recognizable Rep-binding sequences of the CMG. Four putative ORFs can be discerned in the satDNA-III genome, two in the sense and two on the complementary sense (Fig. 5.4b). The relatively larger ORF (V1) is potentially capable of coding for a protein of 75 aa and the smallest ORF C2 a protein of only 26 aa.

5.3.6 Effect of satDNA-II on CMG disease symptom expression

When *N. benthamiana* was inoculated by satDNA-II together with ACMV-[CM] or EACMCV-[CM], symptoms produced were indistinguishable from those induced by the geminviruses alone. However, cv. 60444 plants co-inoculated with satDNA-II and ACMV-[CM] displayed more severe symptoms than those inoculated with only ACMV-[CM] (Fig. 5.1f). No symptom enhancement was observed in plants inoculated with a combination of EACMCV-[CM] and satDNA-II. When EACMV-UG2 or ACMV-[CM] was coinoculated with satDNA-II on cv. 60444 plants, systemic symptoms developed at 14 dpi and were more severe compared to those in plants singly inoculated with EACMV-UG2 (Fig. 5.1h). Plants co-infected with ACMV-[CM] and satDNA-II did not show recovery from CMD symptoms over time.

5.3.7 Enhancement of CMG disease by satDNA-III

Plants of *Nicotiana benthamiana* and cassava cv. 60444 inoculated with satDNA-II and satDNA-III were asymptomatic. *N. benthamiana* plants inoculated with ACMV-[CM] alone produced symptoms that were well pronounced 7 days post-inoculation (dpi) and reached a maximum at 14 dpi. Plants were generally stunted and showed severe leaf distortion but later recovered around 21 dpi in a manner typical for plants infected with this type of geminivirus. Similar results were observed when ACMV-[CM] was co-inoculated with satDNA-III. In contrast, cassava plants of cv. 60444 inoculated with ACMV-[CM] and satDNA-III expressed more severe symptoms than those inoculated with ACMV-[CM] alone (Fig. 5.1d, 1e). In addition, plants inoculated with ACMV-[CM] showed symptom recovery beginning at 21 dpi, but those co-inoculated with ACMV-[CM] and satDNA-III displayed no reduction in symptom severity by 65 dpi. *N. benthamiana*, plants inoculated with EACMCV-[CM] alone developed mild symptoms starting at 7 dpi which peaked at about 45 dpi and showed no recovery even within the 3 month observation period (Fig. 5.1j). Challenging plants with EACMCV-[CM] and satDNA-III in combination, induced symptoms as early as 4 dpi which developed to become very severe by 28 dpi and

consisted of stunted growth, severe downward leaf curling and shortening of internodes, which imparted a rosette appearance to the top, leaves. Similarly, cassava plants of cv. 60444 inoculated with EACMCV-[CM] alone produced only mild systemic symptoms 20 dpi whereas those co-inoculated with satDNA-III produced severe systemic mosaic symptoms as early as 7 dpi (Fig. 5.1g).

Fondong *et al*., (2000) and Pita *et al*., (2001) have reported the inability of biolistic inoculation to transmit EACMV-Uganda strain (EACMV-UG2) to *N. benthamiana* with all plants inoculated with EACMV-UG2 alone remaining asymptomatic. When the same virus was coinoculated with satDNA-III in the present study, moderate systemic mosaic symptoms were observed at 7 dpi with a maximum severity at 21 dpi suggesting that satDNA-III can help EACMV-UG2 to establish disease and express symptoms in *N. benthamiana*.

5.3.8 Breaking of TME3 natural resistance by satDNA-II and satDNA-III

Cassava plants were biolistically co-inoculated with ACMV-[CM], EACMCV-[CM] or EACMV-UG2 in combination with either satDNA-II or satDNA-III. TME3 displayed high levels of resistance to inoculation with EACMCV-[CM] or EACMV-UG2 with no symptoms developing on symptomatic leaves. However, TME3 plants inoculated with ACMV-[CM] in combination with satDNA-II or satDNA-III developed mild mosaic, which lessened on each newly developed leaf and disappeared by 35 dpi after which the plants remained asymptomatic in a manner similar to an inoculation without the satDNAs. However, plants co-inoculated with EACMV-UG2 and satDNA-II developed severe mosaic symptoms 21 dpi, which were maintained for up to eight months after inoculation. CMD symptoms on these plants were distinct, comprising predominantly yellow bleaching of the leaves mainly along the veins (Fig. 1l). Plants inoculated with EACMV-UG2 alone showed a slight chlorotic blotch 14 dpi on inoculated leaves but the symptoms did not become systemic (Fig. 5.1k). Co-inoculation of EACMCV-[CM] and satDNA-II resulted

in mild systemic mosaic symptoms around 45 dpi. Plants inoculated with EACMV-UG2 and satDNA-III in combination developed systemic mosaic symptoms 60 days after inoculation, whereas plants singly inoculated with satDNA-III or EACMV-UG2 were asymptomatic. The presence of satDNA-II and EACMV-UG2 in the systemic infected tissue was confirmed by Southern blot analysis and that of EACMV-[CM] and satDNA-III in TME3 and cv. 60444 by PCR.

5.3.9 Effects of satDNA II and satDNA III on the accumulation of CMG DNA

The experimental host *N. benthamiana* the cassava CMD resistant landrace TME3 and the susceptible cv. 60444 were used to study the effect of satDNA-II and satDNA-III on ACMV-[CM], EACMCV-[CM] and EACMV-UG2 accumulation. Systemic leaves were collected from *N. benthamiana* (14 dpi) and cassava (21 dpi) for total DNA extraction. Southern blot analysis was carried out using probes specific for ACMV-[CM], EACMCV-[CM], EACMV-UG2, satDNA-II and satDNA-III. *Nicotiana benthamiana* plants inoculated with ACMV-[CM] alone or in combination satDNA-II or satDNA-III showed no noticeable difference in viral accumulation (results not shown). In contrast, *N. benthamiana* plants inoculated with both EACMCV-[CM] and satDNA-III, displayed higher viral accumulation of EACMV-[CM] compared to plants inoculated with EACMCV-[CM] alone (Fig. 5.5a) consistent with the enhanced systemic symptoms observed in the presence of satDNA-III. However, the presence of satDNA-II in plants inoculated with EACMCV-[CM] did not result in higher accumulation of EACMCV-[CM] and correlated with the failure of this satDNA-II to enhance disease symptoms. In *N. benthamiana*, EACMV-UG2 levels were undetectable when inoculated with EACMV-UG2 alone but typical DNA forms indicative of replication were evident when it was inoculated together with satDNA-III (Fig. 5.5b). The results of Southern blot analysis of systemically infected leaves of the cassava landrace TME3 inoculated with EACMV-UG2 alone showed no detectable signal at 21 dpi. However, replicative forms of EACMV-UG2 were detected in the systemically infected leaves of plants coinoculated with EACMV-UG2 and satDNA-II (Fig. 5.5a).

Southern blot analysis also confirmed the presence of DNA replicative forms of satDNA-II and satDNA-III in the original cassava samples collected from the farmer's fields in Tanzania (Fig. 5.6a). The satDNA-II and satDNA-III replicative intermediates were only detected in the leaves of plants inoculated with satDNA-II or satDNA-III in the presence of the CMG (Fig. 5.5b) confirming that replication and systemic spread of satDNA-II and satDNA-III is dependent on the presence of the helper viruses.

5.4 DISCUSSION

We have demonstrated that some cassava plants growing in Tanzania, which exhibit mosaic symptoms induced by bipartite CMG are infected with single-stranded DNA satellites that we refer to as satDNA-II and satDNA-III. SatDNA-II and satDNA-III show no significant sequence similarity to known geminivirus genome components DNA-A or DNA-B and are unrelated to other known DNA satellites found associated with monopartite begomoviruses. They appear, therefore, to represent a distinct group of satellites, perhaps adapted to bipartite begomoviruses. The appearance of satDNA-II and satDNA-III in the upper systemic leaves of naturally or artificially infected plants suggests that they can be replicated and mobilized from the site of inoculation by the helper viruses in a manner similar to satellite molecules associated with some RNA viruses (Collmer and Howell, 1992). In the cassava-infecting bipartite begomoviruses ACMV-[CM], EACMCV-[CM] and EACMV-UG2 used in this study, systemic spread is achieved through specific movement proteins encoded on the DNA-B component, a process which is highly conserved in plants (Sanderfoot *et al*., 1996; McGarry *et al*., 2003). Thus, satDNA-II and satDNA-III may possess, as yet unidentified, sequence elements to allow for interaction with helper geminiviruses. The mode of transmission of these two satellites to cassava plants remains to be determined but it is probable that they are co-transmitted by whiteflies with the CMG particles as shown for other satellites of monopartite begomoviruses (Mansoor *et al*., 2003). Their size of 1.0 and 1.2 kbp is close to half geminivirus genome size and therefore compatible with the encapsidation with the geminivirus helper capsid protein.

Recovery of these satDNA sequences with beta01 and DNA1/F primers is unexpected as was the fact that apparently only one primer has been used in the amplification process. However, amplification with a single primer has been reported in other cases and may reflect the specific organization of these satDNAs. Maruthi (2001) reported a similar situation when part of DNA-B of East African cassava mosaic Zanzibar virus had a 244 bp upstream of the 5′ of BV1 ORF duplicated and inserted in a reverse orientation towards the 3′ end of intergenic region. The fact that we obtained in the amplified sequence the exact same primer sequence in opposite orientation may be explained by the fact that one of the primers had a high homology with the satDNA sequence and consequently the sequence obtained in this region may not represent exactly the reality. However, the fact that these molecules could be amplified from cassava plants exhibiting particular symptoms in the field and not from healthy plants, and that they induced the biological properties listed in this paper call for the existence of such satellites in nature, if not with the exact same sequence, at least close enough to retain these unique biological properties.

Both satDNA-II and satDNA-III contain a region of high GC content. At present the role of these regions is unknown, although such GC-rich sequences are known to be resistant to methylation and to be associated with the promoters and with frequently expressed genes (Burns *et al*., 1995). Although the two satellite molecules described here have only 24% identity based on overall nucleotide sequence comparison, their GC-rich region showed 51% nucleotide identity, suggesting a possible common but as yet unknown function performed by this region. It is most important, therefore, to determine what functions this region may have and how they interact with the helper virus or plant host-encoded proteins. Since satDNA-II and satDNA-III do not contain known Rep-binding sequences but depend on the CMG for replication, it is possible that different unidentified sequences in these two satellites are recognized by the helper viruses to initiate DNA-A-based trans-replication. The 682 nts satellite DNA of ToLCV depends on its helper virus for its replication but does not utilize a typical Rep-binding sequence of the helper for replication (Lin *et al*., 2003) suggesting a different type of interaction.

The satDNA-II and satDNA-III sequences suggest that at least some of the ORFs encode for possible functional proteins. Both satellite molecules contain at least one putative TATA box; polyadenylation signal (AATAAA) and other putative promoter associated regulatory elements (Hanley-Bowdoin *et al*., 1999; Paran *et al*., 2000;). The significance of the presence of various short sequences (13 to 33 nts) with high identity too many common sequences among a variety of organisms remains to be understood.

SatDNA-II did not modify symptom phenotype in *N. benthamiana* or cassava cv. 60444 when inoculated in combination with EACMCV-[CM] and there was no detectable change in helper virus DNA accumulation in the presence of this satellite. This result suggests that satDNA-II is incapable of exerting its biological functions when EACMCV-[CM] is the helper virus. Alternatively, the biological effects of satDNA-II may be counteracted by the plant host or helper virus encoded factors. It is tempting to suggest interaction with the host, because satDNA-II is able to exacerbate pathogenicity of EACMV-UG2 and EACMCV-[CM] in cassava landrace TME3, resulting in a dramatic increase in symptom expression by the helper virus and increased accumulation of viral genomic components.

Mechanisms by which satDNA-II and satDNA-III overcome virus resistance in TME3 remains unclear but our analysis shows a direct correlation between CMD symptom appearance and increased EACMV-UG2 accumulation in the presence of satDNA-II. To account for the increased symptoms and levels of viral DNA that occurred in TME3 when EACMV-UG2 was coinoculated with satDNA-II, one hypothesis may be that satDNA-II acts as a replication enhancer. In doing so it may manipulate the cellular environment making it favorable for virus replication, possibly by overcoming host defense mechanisms such as gene silencing, gene transcription or messenger translation. It can also be suggested that perhaps some of the putative ORFs of the satellite molecules are capable of encoding for pathogenicity factors involved in symptom enhancement as reported recently for symptom-modulating by DNA-β (Saunders *et al*., 2004). Alternatively, by analogy to the function of DNA-B proteins in bipartite begomoviruses, which encode two gene products involved in virus movement (Noueiry *et al*., 1994), the product(s) encoded by

satDNA-II may be involved in cell-to-cell movement. A similar hypothesis can be used to explain the ability of satDNA-III to exacerbate symptoms produced by EACMCV-[CM] and EACMVU-G2 in *N. benthamiana* and the observed increase in viral accumulation. Consistent with this, cv. 60444 cassava plants co-inoculated with CMGs (EACMCV-[CM], ACMV-[CM], or EACMV-UG2) and with satDNA-III displayed more severe symptoms than those inoculated with either of these viruses alone, suggesting interaction between the CMG and satDNA-III, regardless of the host type. The symptoms caused by EACMV-UG2 in TME3 in the presence of satDNA-II were mainly yellow mosaic characterized by yellow bleaching along the veins.

The resistance breaking of satDNA-II and satDNA-III in TME3, in a single cycle of inoculation, is extremely puzzling relative to the mode of action of these satellites and will constitute an excellent tool to study the mechanism involved. It is also indicating a very complementary role of the satellites to the CMGs that become fully capable of infecting that particularly resistant genotype in normal conditions.

Although we do not fully understand the molecular nature of satDNA-II and satDNA-III, including certain aspects of their sequence, they were found only in diseased plants, thereby excluding the possibility that they are PCR artifacts. Furthermore, it was proven that they are biologically functional, are replicated and systemically moved by CMGs, they enhance CMG symptoms in susceptible plant hosts and above all they can break very high levels of resistance to CMD in the cassava landrace TME3, thereby indicating the involvement of a completely different mechanism in the host-pathogen interactions.

Elucidation of the mechanisms by which satDNA-II and satDNA-III interact with both the helper virus and the host plant to produce severe systemic symptoms may help us to better understand the intimate molecular relationships between viruses and other molecular mechanisms in the plant cell machinery. The fact that satDNA-II enhances symptoms of CMGs in cv. 60444 and TME3 but not in *N. benthamiana* suggests involvement of specific host factors in the symptom exacerbation. The presence of these newly characterized

CMG satellites may also shed light on the causal effects of the cassava pandemic presently occurring in Africa. At this time, the pandemic of severe cassava mosaic disease in Eastern and Central African countries has mainly been attributed to synergism, recombination and pseudo-recombination involving EACMV, EACMV-UG2 and ACMV DNA genomes. The discovery of satellite molecules associated with CMGs in more than 25 samples in a region where the pandemic occurs and their ability to exacerbate disease symptoms provides possible additional explanations of this pandemic. Investigation of the possible role of the satDNA-II and satDNA-III and their spread in the epidemiology of CMGs in Eastern and Central African countries, especially in the Lake Victoria basin, is urgently needed. In addition, the fact that these molecules can break the CMD resistance of TME3, an important source of resistance for cassava breeding programs in Africa, is of a concern and merits further investigation.

5.5 KNOWLEDGEMENTS

This study was funded by UK Department for International Development (DFID) through the International Institute of Tropical Agriculture (IITA) that granted the fellowship to the senior author.

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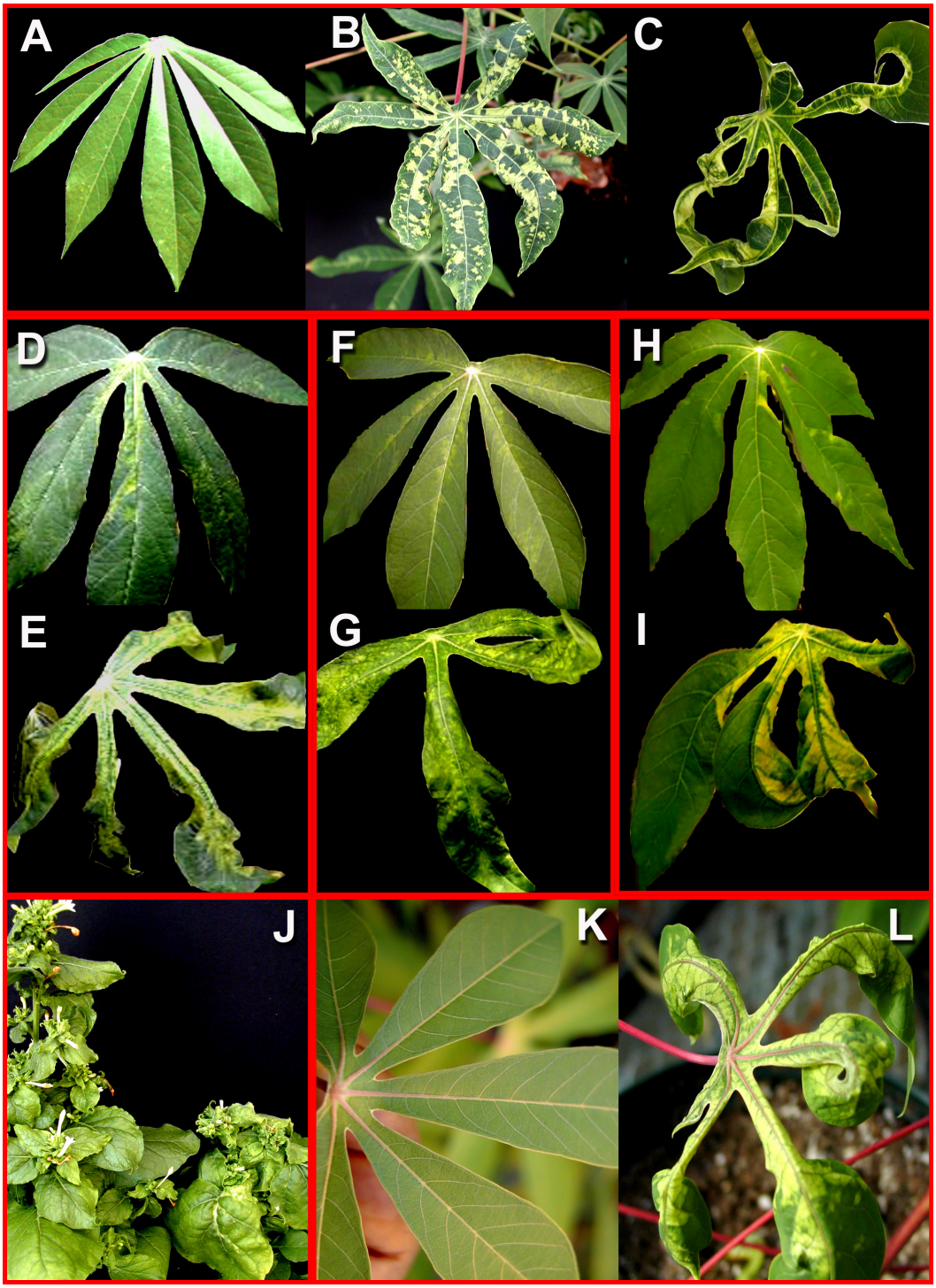

Fig. 5.1. Symptoms caused by the association of DNA satellites to cassava mosaic geminiviruses. Pictures (a-c) represent close-up leaf symptoms from plants imported from

Tanzanian fields, while (g-l) represent symptoms of plants inoculated in the laboratory with DNA infectious clones using particle bombardment.

(a) Healthy plant from a Tanzanian cassava cv.; (b) leaf from a Tanzanian cassava cv. in which satDNA-II was detected; (c) leaf from a Tanzanian cassava cv. in which satDNA-III was detected.

(D-I) CMD symptoms on cassava cv. 60444 7dpi inoculated with different combinations of geminiviruses and satellites: (d)- ACMV-[CM]; (e)- ACMV-[CM] + satDNA-II; (f)- EACMCV-[CM]; (g)- EACMCV-[CM] + satDNA-III; (h)- EACMV-UG2, (i)- EACMV-UG2 + satDNA-II.

(j) *Nicotiana benthamiana* plants inoculated with the combination EACMCV-[CM] DNA-A and DNA-B (left) and the combination EACMCV-[CM] DNA-A and DNA-B plus satDNA-III (right), 35 dpi;

(k-l) virus resistant cassava landrace TME3 inoculated with the combination: (k)- EACMV-UG2 DNA-A and EACMV-UG3 DNA-B; (l)- EACMV-UG2 DNA-A and EACMV-UG3 DNA-B plus satDNA-II, 60 dpi.

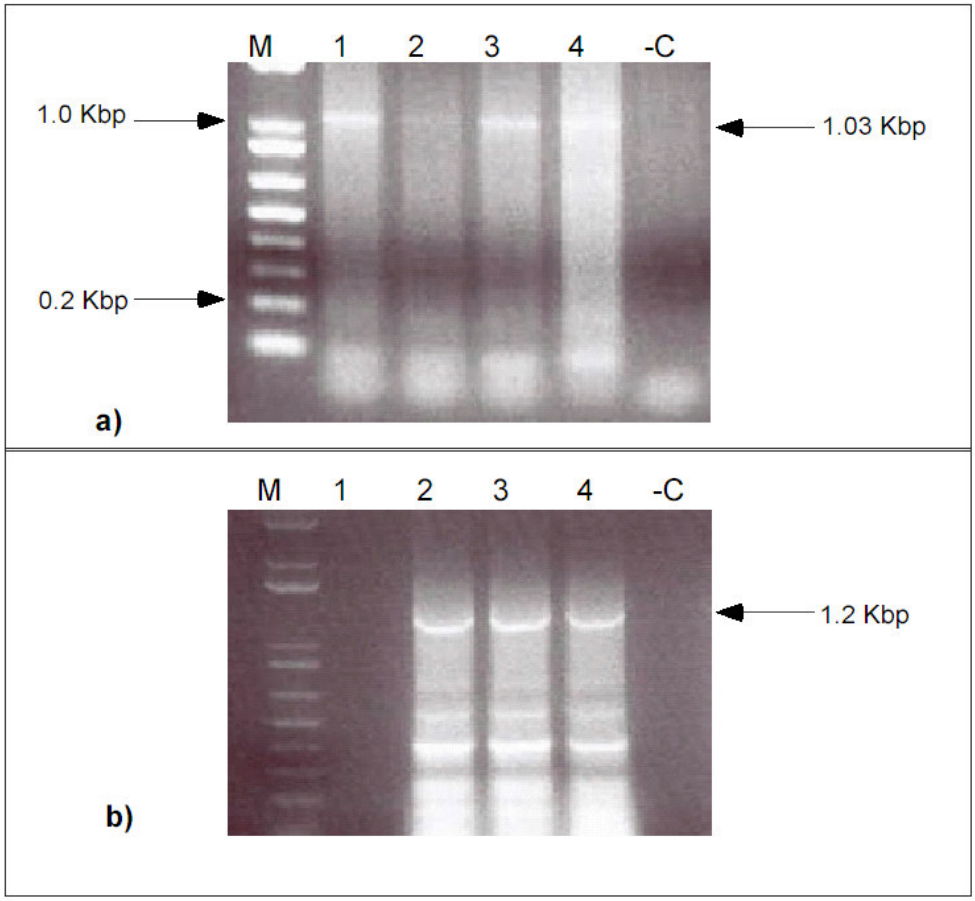

Fig. 5.2. PCR detection of cassava mosaic virus-associated satellite DNA molecules. Total DNA was extracted from leaves of field-collected cassava where mosaic symptoms were observed. (a) satDNA-II; (b) satDNA-III. SatDNA-II and satDNA-III were amplified using the primer pairs Beta01/Beta02 and DNA1-F/DNA1-R respectively; lanes 1-4 represent samples for infected symptomatic cassava plants; (-c) represents a sample from healthy control cassava; M: DNA marker; Arrows on the right indicate the expected fragment for satDNA-II (1.03 kbp) and satDNA-III (1.2 kbp); Lane b1 represents a CMG-infected plant but which is PCR-negative for satDNA-III. kbp: kilo base pair.

Beta01 (5´-3´)


GGTACCACTACGCTACGCAGCAGCCATCATCGACATCGTATTTTAACCAG 50
AGGACCCGTCGACCGCCTGAGCAGCAGCACGTCGCACCAGCACCACCGCC 100
GCATCGCGCGCCTGTGAGCCGCCGCACCACTGGATCTCGTGCTCGTGAGC 150
CGCCGCACGCCGCAACTCTTCATCTACCGCTCGTTTACAGCCCACCTCTG 200
TATCACGCGATTGTGAGCCGCCGACTGCCCGCCGCACGCCCGCACCTCTG 250
CATCAACTGCTCGTTTGCCACCCACCTCGCTCCTCTGCAGTTCAGCAGTT 300
CAACTGTAAGCATTTTTTCGTTAAATCTGAAGAAAATAGTTCTGGATAGA 350
ATTTTGATTGGTAAGCATTATGAATTTATTATGACATTCAAGTTTATA GG 400
CATCATAGTGTTGCTTAGGACATACTTAGCTTGTAGTTCCAGAAAATAGA 450
GTCATTTCTGGTTTTCTTTTACAATGGAGGTGTTTATTCCATTGTAATT 500
TGAGCTGAGCTTTGTTAAGGACCTTTGGAGCTCGAGCTTTGTTTACAAGG 550
CATCTTGATAGAGCTTTTCGAGCTCGAATTAGAATTAGGCTCATGGTTAT 600
ACTAAAGGGAGTTTTTCATGAGTTTGAGTGCTTCCAAAATTTTTTAATAA 650
AAGCTTTACAAAGCTCAGCTTGGATCGATTACACCTCTACTGACCCTACT 700
CAGTTTGGGACTCTGGCTGGGGCCATTCTCAAAAGCCATTTATCTGGGTA 750
GCCTCTAATCCTTCAACTCTATTTTTCCGTTTGGTTCTGAGAGAGTACTA 800
AAAAGGAAATCCAACCATATATGATCAAATCTAATGATATAGCTGGTGAG 850
TACTGCAACATAATTGCAATTTATGCAGTTATTTCTCTTGAATTTGGTAT 900
CTGCAATTTATGTATAAATCCCTAGCAGAATATTTTACTGGAGTGGTGAA 950
TATGTGTAGGCTTCACTATGGTGGAAATGGAAATTTGTGTGTGATAACTT 1000
CCTGACTGGCTGCTGCGTAGCGTAGTGGTACC 1032
Beta01 (5´-3´)

a)

b)

Fig. 5.3. Complete nucleotide sequence of cassava mosaic virus associated satellites DNA molecules. (a) satDNA-II and (b) satDNA-III. The region of high GC composition in both satellites is boxed. Direct repeat sequences are shaded black. A consensus putative transcript polyadenylation signal (AATAAA) is indicated by gray shade with an underline. The putative TATA boxes in the two satellites are shaded gray. The putative TTGTA motif

frequently associated with functional polyadenylation signals (Rothnie *et al*., 1994) is positioned upstream of the polyadenylation signal shown in bold and boxed. For satDNA-III, the putative ACGTCA motif believed to be associated with strong promoter activity in undifferentiated actively dividing cell types (Nakayama *et al*., 1992) is indicated in bold and underlined. The predicted AP-1 binding motifs are underlined. The position of primers Beta01 and DNA1-F used for amplification of satDNA-II and satDNA-III, respectively, is indicated.

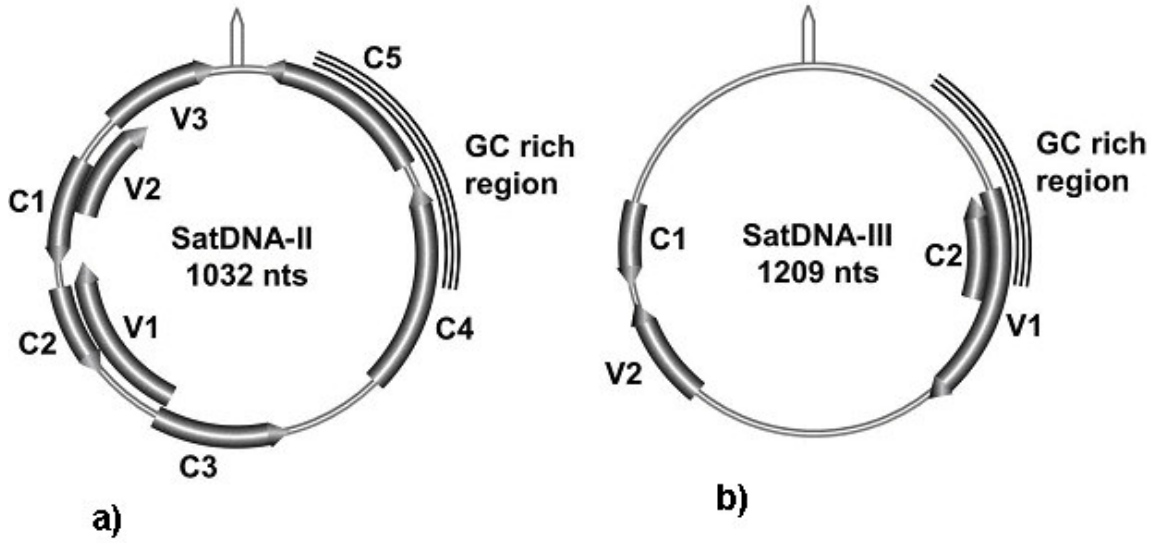

Fig. 5.4. Genome organization of cassava mosaic geminivirus-associated satellites DNA molecules. (a) satDNA-II; (b) satDNA-III. The putative genes encoded on either virion-sense (V) or complementary sense (C) are indicated by dark arrows and their direction of transcription is shown. The name of each open reading frame is shown in bold type. The hatched regions represent the GC-rich region present in both satDNA-II and satDNA-III.

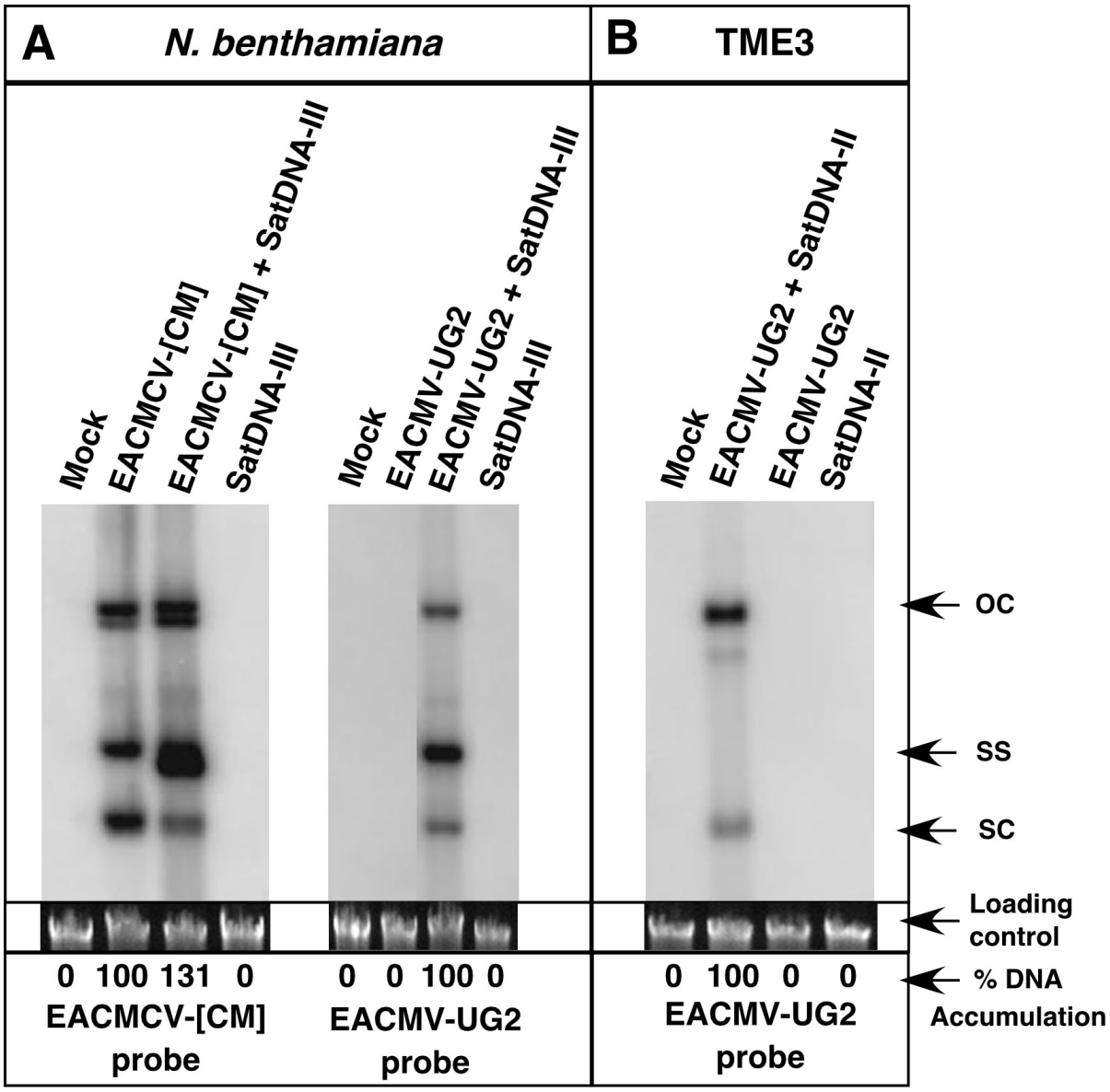

Fig. 5.5. Accumulation of viral DNA in systemic leaves of *Nicotiana benthamiana* and cassava plants of landrace TME3. Southern blots show detection of EACMCV-[CM], EACMV-UG2, satDNA-II and satDNA-III using specific probes.

(a). *N. benthamiana* plants inoculated with the combinations (EACMCV-[CM] DNA-A and DNA-B), (EACMV-UG2 DNA-A and EACMV-UG3 DNA-B), satDNA-II and satDNA-III alone or in combination;

(b). TME3 plants were inoculated with the combination (EACMV-UG2 DNA-A and EACMV-UG3 DNA-B) and satDNA-II alone or in combination.

Samples were collected at 14 days post-inoculation (dpi) for *N. benthamiana* and 21 dpi for TME3, inoculated with viral DNA infectious clones. DNA accumulation was quantified and the values indicated under each lane for each blot refer to the percentage of viral DNA amount.

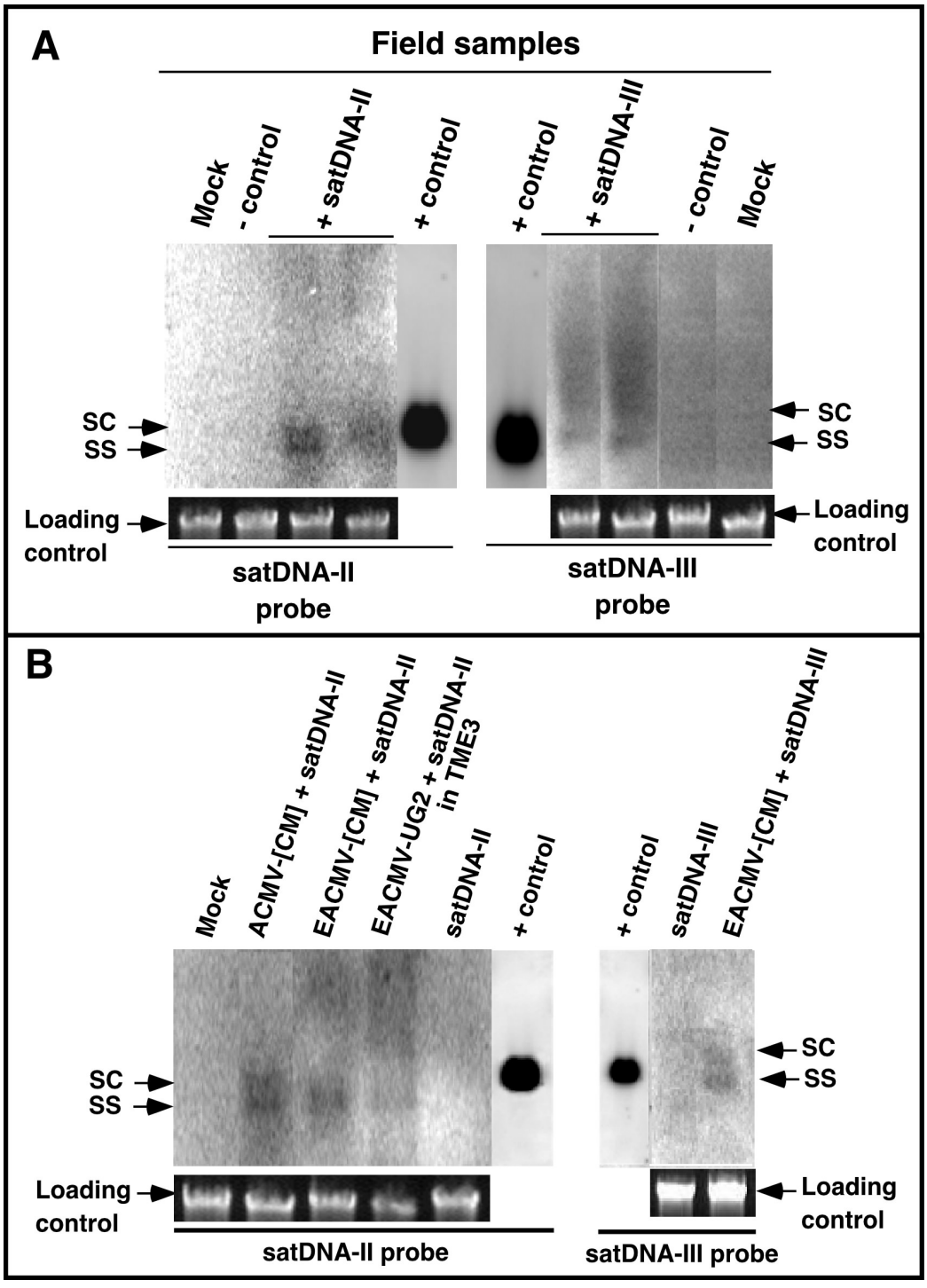

Fig. 5.6. Accumulation of viral DNA in systemic leaves of *Nicotiana benthamiana* and cassava plants. Southern blots show detection of EACMCV-[CM], EACMV-UG2, satDNA-II and satDNA-III using specific probes.

(a) accumulation of satDNA-II and satDNA-III in cassava field samples. – control corresponds to DNA samples from CMD-afftected cassava plant that tested negative for satDNA-II and satDNA-III

(b) accumulation of satDNA-II and satDNA-III in *N. benthamiana* and TME3 inoculated with viral DNA infectious clones. Only one sample corresponds to DNA from TME3 and is indicated on the figure. + control corresponds to full-length satDNA-II and satDNA-III digested from plasmid infectious clone.