Differential interaction between cassava mosaic geminiviruses and geminivirus satellites

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Geminiviruses are often associated with subviral agents called DNA satellites that require proteins encoded by the helper virus for their replication, movement and encapsidation. Hitherto, most of the single-stranded DNA satellites reported to be associated with members of the family Geminiviridae have been associated with monopartite begomoviruses. Cassava mosaic disease is known to be caused by viruses belonging to nine different begomovirus species in the African continent and the Indian subcontinent. In addition to these species, several strains have been recognized that exhibit contrasting phenotypes and infection dynamics. It is established that Sri Lankan cassava mosaic virus can trans-replicate betasatellites and can cross host barriers. To extend these studies further, we carried out an exhaustive investigation of the ability of geminiviruses, selected to represent all cassava-infecting geminivirus species, to trans-replicate betasatellites (DNA-b) and to interact with alphasatellites (nanovirus-like components; previously called DNA-1). Each of the cassava-infecting geminiviruses showed a contrasting and differential interaction with the DNA satellites, not only in the capacity to interact with these molecules but also in the modulation of symptom phenotypes by the satellites. These observations could be extrapolated to field situations in order to hypothesize about the possibility of acquisition of such DNA satellites currently associated with other begomoviruses. These results call for more detailed analyses of these subviral components and an investigation of their possible interaction with the cassava mosaic disease complex.

INTRODUCTION

Plant viruses are often associated with satellite DNA molecules, which are, in most cases, able to modulate replication and symptom expression of their helper virus (Roossinck et al., 1992; Simon et al., 2004). These satellite molecules completely lack sequence identity to their helper viruses and depend on the helper virus for all or some of the following functions: replication, movement, encapsidation and transmission. Satellite molecules were initially reported to be associated with RNA viruses; these satellites are very well characterized (Simon et al., 2004). In the last decade, more than 500 satellite sequences associated with begomoviruses (family Geminiviridae) have been isolated from a diverse range of cultivated crops and weeds (Briddon & Stanley, 2006; Briddon et al., 2008).

Two such classes of DNA satellite are associated with several monopartite begomoviruses, namely alphasatellites (formerly called DNA-1) and betasatellites (formerly called DNA-β) (Briddon et al., 2008; R. W. Briddon, B. Gronenborn, J. Vetten, X. Zhou & C. M. Fauquet, unpublished data). In addition to these subviral components, geminiviruses have been shown to be accompanied by smaller-sized DNA molecules, called defective DNA molecules, that are derived from the helper virus genomes, but are not satellite molecules (Patil & Dasgupta, 2006; Simon et al., 2004). Alphasatellites are 1.3 kb nanovirus-like components that, in some cases, suppress viral disease symptoms. Although alphasatellites encode a replication-associated protein, they depend on the helper virus-encoded proteins for movement and encapsidation (Briddon & Stanley, 2006; Nawaz-ul-Rehman & Fauquet, 2009). The first alphasatellite components identified were associated with cotton leaf curl disease (CLCuD) and Ageratum yellow vein disease (AYVD) complexes. Since then, many similar molecules have been reported to be associated with a diverse range of begomovirus disease complexes (Briddon et al., 2004; Mansoor et al., 2001; Saunders & Stanley, 1999).

Betasatellites are a diverse set of symptom-enhancing, single-stranded DNA (ssDNA) molecules that are 1.3 kb in size and only associated with monopartite begomoviruses from the Old World (OW), namely Asia and Africa (Briddon et al., 2001, 2003, 2008; Bull et al., 2004; Mansoor...
et al., 2003; Zhou et al., 2003). Most recently, betasatellites have been found associated with a few bipartite begomoviruses (Rouhibakhsh & Malathi, 2005). They have been shown to play a direct role in symptom enhancement with characteristic stem-curling and vein-swelling phenotypes, impacting host-range determination and facilitating accumulation of both the begomovirus molecules and the encoded pathogenicity factors (Mansoor et al., 2006; Saunders et al., 2002, 2004; Stanley, 2004).

Cassava mosaic disease (CMD) is known to be caused by viruses belonging to seven different bipartite begomovirus 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), East African cassava mosaic Kenya virus (EACMKV) and South African cassava mosaic virus (SACMV)], and to two species in the Indian subcontinent [Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV)] (Fauquet et al., 2008). Each of these virus species consists of two subgenomic DNA components, DNA-A and DNA-B, and they are known to exhibit significantly diverse infection dynamics in terms of symptom progression, recovery and severity, and host range (Bull et al., 2007; Patil & Fauquet, 2009). Earlier studies on the ability of the cassava mosaic geminiviruses (CMGs) to trans-replicate satellite DNA molecules were done for ICMV, SLCMV and ACMV (Saeed et al., 2007; Saunders et al., 2002).

To extend these studies further and to investigate the possibility of acquisition of such satellite molecules by other CMGs in the field, we carried out a systematic and detailed study for eight CMGs from the Indian subcontinent and Africa (with the exception of EACMMV) in the model host Nicotiana benthamiana. We also extended these studies to two New World (NW) begomoviruses [cabbage leaf curl virus (CabLCuV) and tomato golden mosaic virus (TGVM)], a curtovirus [bean curly top virus (BCTV)] and a mastrevirus [bean yellow dwarf virus (BeYDV)] with both betasatellites and alphasatellites. A possible mechanism of symptom suppression by alphasatellites has also been hypothesized.

RESULTS

Trans-replication of betasatellites and their role in symptom severity

The Ageratum yellow vein betasatellite (AYVB), associated with the AYYD complex, was used to study the nature of betasatellite interaction with CMGs in N. benthamiana. The experiments showed that six of the CMGs could trans-replicate AYVB (indicated as a strong interaction in Table 1) and that two viruses, EACMCV and EACMCV, could not (no interaction, Table 1). The NW begomoviruses CabLCuV and TGVM and the mastrevirus BeYDV failed to trans-replicate the betasatellite, whilst BCTV supported a small amount of AYVB trans-replication with no effect on its symptom phenotype. Betasatellites enhanced disease symptoms and caused the early initiation of symptoms for five of the trans-replicating CMGs (ICMV, ACMV, SACMV, EACMKV and EACMZV), but did not alter disease progression for SLCMV, a highly virulent virus causing necrosis in N. benthamiana. The typical stem-curling phenotype produced by betasatellites was observed for all CMGs trans-replicating AYVB (Fig. 1; Supplementary Fig. S1, available in JGV Online).

In order to study the effect of betasatellites on helper virus replication, we studied the evolution over time of the symptoms induced on N. benthamiana (Fig. 2), followed by a quantification of the accumulation of the helper viruses and betasatellites (Figs 3 and 4).

To understand further whether CMGs specifically trans-replicate only certain betasatellites, we co-inoculated the eight CMGs with two other betasatellites, honeysuckle yellow vein betasatellite (HYVB) and Eupatorium yellow vein betasatellite (EpYMVB). The studies showed that all CMGs exhibited a similar type of behaviour in trans-replicating the betasatellites and that the betasatellites had the same symptom-modulation effect, demonstrating that the interaction of CMGs was similar for all three betasatellites tested (data not shown). The accumulation of the betasatellites, as a result of trans-replication by the CMG helper viruses, was confirmed by both PCR analysis and Southern hybridizations (Fig. 3).

Infected plants were analysed for accumulation of all components (DNA-A, DNA-B, betasatellite and alphasatellite) both by PCR (not shown) and by Southern hybridizations at 1 and 3 weeks post-inoculation (p.i.) (Figs 3 and 4). The Southern hybridizations indicate that the CMGs ICMV, SLCMV, ACMV, SACMV, EACMKV-K229, EACMZV-K275 and EACMV-K19 trans-replicated the betasatellites, thus yielding a successful symptomatic infection when the betasatellite was substituted for the helper virus component, DNA-B. Most of the CMGs that trans-replicated the betasatellites showed a significant increase in accumulation of betasatellite and a decrease in the titre of the helper virus components in the absence of the DNA-B component, in contrast to the presence of both DNA-B and betasatellite (Fig. 3d, f–j). The betasatellite was not detected by Southern hybridizations in the cases of co-inoculations with EACMV-K24 (Fig. 3a), -K201 (Fig. 3b), -Ug-K282 (not shown), -Ug2A (not shown) and EACMCV (Fig. 3c), thus indicating that two CMG species, EACMV and EACMCV, failed to trans-replicate the betasatellite (Fig. 3a–c). In most of the inoculations, the DNA-B component produced defective molecules, which was aggravated in the presence of betasatellites (data not shown).
DNA-A (AYVB, HYVB or EpYMVB). The symptoms in all cases of also yielded infection and symptoms when the DNA-B trans complementation of virus infectivity and movement functions

Interaction between cassava geminivirus and satellites

Substitution of DNA-B with betasatellite for the complementation of virus infectivity and movement functions

All six CMGs that could trans-replicate betasatellites also yielded infection and symptoms when the DNA-B component of CMGs was substituted for a betasatellite (AYVB, HYVB or EpYMVB). The symptoms in all cases of DNA-A+betasatellite inoculations (DNA-A+β) were milder and delayed compared with DNA-A+DNA-B inoculum combinations (Figs 1 and 2). However, this was not prominently visible in the case of K19 compared with K275, the two different isolates of EACMZV (Fig. 2). To investigate whether the high initial inoculum of betasatellite (due to agro-infection) had any role in manifesting the characteristic stem-curling and vein-swelling phenotypes, *N. benthamiana* plants were inoculated with different ratios of DNA-A:betasatellite, such as 1:1, 1:2 and 1:3. Interestingly, there was no significant difference recorded in terms of symptom initiation, progression or phenotype produced (data not shown). The expression of the characteristic stem-curling phenotype produced by betasatellites was very strong only in the presence of betasatellite and was absent in the presence of the DNA-B component only (Fig. 1, first two columns).

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Interaction with alphasatellites (AYVA-1)

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Replication, accumulation and effect on symptoms of the Ageratum yellow vein alphassatellite (AYVA) with CMGs

The alphassatellite AYVA-1, associated with the AYVD complex, was used to study the nature of alphassatellite interaction with CMGs in *N. benthamiana*. AYVA-1 accumulated with all CMG combinations (Fig. 3). As reported for other alphassatellite–begomovirus interactions, AYVA-1 suppressed symptoms in some CMG combinations (Table 1) (Saunders & Stanley, 1999). Symptom initiation was delayed significantly and the severity was reduced in some of the CMGs, similarly to the curtovirus BCTV. AYVA-1 exhibited different degrees of symptom suppression, ranging from none (e.g. EACMKV-K229) to strong (e.g. EACMZV-K275) when co-inoculated with different CMGs (Fig. 1). This corresponded with the reduction in the titre of helper virus components (Figs 3 and 4). Whilst the alphassatellite AYVA-1 had no or only a mild initial symptom suppression effect on EACMKV-K229 (Fig. 2g) and SACMV (data not shown), the later symptoms were more severe than the DNA-A+DNA-B combination (red line), which eventually recovered. AYVA-1 had a strong symptom-suppressing effect on ICMV (not shown) and EACMZV-K275 (Fig. 2c), whereas EACMZV-K19 was suppressed only mildly by this

**Table 1.** Nature of interactions (symptom enhancement or suppression) between geminiviruses and DNA satellites as interpreted from the trans-replication and accumulation of the DNA betasatellites (AYVB, HYVB and EpYMVB) and the alphassatellite (AYVA-1) and their effect on symptom phenotype when co-inoculated with OW cassava-infecting geminiviruses or with representatives of NW begomoviruses, curtovirus or mastrevirus in *N. benthamiana*.

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alphasatellite (Fig. 2d). ACMV, SLCMV, EACMCV and several isolates of EACMV (K24, K201 and Ug-K282) did not show any delay in symptom initiation or reduction in symptom severity in the presence of the alphasatellite AYVA-1, although it accumulated to very significant levels (Figs 2e, f and 3). Surprisingly, in the case of EACMV-K201 co-inoculated with AYVA-1, there was no mild recovery, in contrast to the partial recovery obtained with the inoculation of DNA-A+DNA-B components in *N. benthamiana* (Fig. 2e).

Southern analysis showed that the alphasatellite AYVA-1 could accumulate when co-inoculated with all of the CMG clones, although symptom suppression was not observed with all viruses. Despite the previously reported autoreplication capacity of the alphasatellite AYVA-1, this alphasatellite was not detected when co-inoculated with the NW geminivirus CabLCuV (data not shown), which is not well-adapted to *N. benthamiana*. For the CMG combinations with the alphasatellite, the 1 week p.i. Southern hybridizations show a significant reduction in the amount of both of the helper virus components (DNA-A and DNA-B), irrespective of the type of symptom phenotype obtained, in most cases (data not shown). After 3 weeks p.i., the titre of the helper virus components had increased significantly and was on par with the levels of accumulation obtained in the absence of AYVA-1. Among the two EACMZV isolates studied, the response to AYVA-1 of EACMZV-K275 was very strong, whilst the response by EACMZV-K19 was moderate.

**Fig. 1.** Symptom phenotypes obtained in *N. benthamiana* when a CMG was co-inoculated with either a betasatellite (β) or an alphasatellite (α) associated with AYVV. Photographs were taken at 1 and 3 weeks post-inoculation (p.i.) for the following four combination of inoculations (shown in columns): CMG DNA-A + DNA-B; CMG DNA-A + β; CMG DNA-A + CMG DNA-B + β; and CMG DNA-A + CMG DNA-B + α. Symptoms of the four combinations at 1 and 3 weeks p.i. are shown for (a) EACMV-KE[KE : Kat : K24 : 01]; (b) EACMKV-[KE : Keh : K229 : 02]; (c) EACMZV-[KE : Kib : K275 : 02].

**Differences between NW and OW viruses in their interaction with DNA satellites**

The two NW begomoviruses CabLCuV and TGMV were studied for their interaction with AYVD satellites in *N. benthamiana*, whilst CabLCuV was also tested in *Arabidopsis*. The DNA satellites neither replicated/accumulated with these viruses nor produced an effect on symptom severity with co-inoculation with a NW virus.
Fig. 2. Graphical representation of the infection dynamics of different CMGs co-inoculated with AYVV DNA satellites based on their symptom severity score. (a, b) Differential interaction of the DNA-A component of three CMGs with either the corresponding DNA-B or (a) the betasatellite AYVB, or (b) the alphasatellite AYVA-1. (c–g) Interaction of either DNA-A or DNA-B of (c) EACMZV-K275, (d) EACMZV-K19, (e) EACMV-K201, (f) EACMV-K24 or (g) EACMKV-K229 with AYVB and AYVA-1.
However, most of the CMGs belonging to the OW, except EACMV and EACMCV, trans-replicated the betasatellites.

**Phylogenetic analysis and sequence alignment**

The amino acid sequences of the replication-associated protein (Rep) of the nine CMGs used in this study were analysed by the CLUSTAL W program of DNASTAR in order to draw a phylogenetic tree, using the Rep sequence of the NW begomovirus CabLCuV as an outgroup. The Rep sequences of all CMGs interacting with the betasatellites, ICMV, SLCMV, ACMV, SACMV, EACMKV and EACMZV, grouped into one cluster, whereas the two CMG species that do not interact with the DNA satellites, EACMV and EACMCV, formed a different cluster, along with a CMG not tested in this study, EACMMV (Fig. 5a). Comparison of the aligned amino acid sequences of the CMG Rep proteins showed that sequence differences were more prevalent in the N-terminal half of the Rep proteins from the CMGs, and the boxed region (nt 101–134) may indicate potential amino acids interacting with betasatellites (Fig. 5b) (Campos-Olivas et al., 2002).

**DISCUSSION**

A decade since the discovery of the association between ssDNA satellites and geminivirus disease complexes has yielded the more recent recognition of satellite DNAs as an important component of begomovirus disease complexes, playing an important role in virus pathogenesis (Briddon & Stanley, 2006). The study reported here was carried out in the wake of the recent isolation and identification of disease resistance-breaking satellite DNA molecules associated with CMD in Tanzania (J. Ndunguru and C. M. Fauquet, unpublished data). Thus, this study was designed to investigate the possible existence of such satellite molecules associated with other geminiviruses and whether satellites extend the range of host crops by CMGs. Using

![Fig. 3.](image-url)
representative isolates of eight different CMG species, we investigated the trans-replication of well-characterized betasatellites (AYVB, HYVB and EpYMVB) and the accumulation of the nanovirus-like component called alphasatellite (AYVA-1), and analysed the effect of both types of satellite on symptom phenotype. We also attempted to investigate and reconfirm the trans-replication of satellite DNAs with the NW geminiviruses TGMV and CabLCuV and the curtovirus BCTV (Nawaz-ul-Rehman et al., 2009; Saunders et al., 2002).

These studies show that representatives of each of the CMG species and strains interact differentially with betasatellites and alphasatellites, producing contrasting symptom phenotypes – either enhancement of the disease symptoms or symptom suppression, respectively. The NW geminiviruses CabLCuV and TGMV failed to trans-replicate the betasatellites, whereas the alphasatellite could not accumulate, even though it encodes its own replication-associated protein. This indicates the differential interaction of each geminivirus in trans-replicating the DNA satellites, despite the fact that high-affinity geminivirus Rep binding is apparently not required for the trans-replication of betasatellites (Dry et al., 1997; Fontes et al., 1994; Lin et al., 2003).

Our data show that betasatellites complement the functions of the DNA-B component of CMGs, producing successful infection in the case of all CMGs that trans-replicate the corresponding betasatellite. These new data in N. benthamiana strengthen the idea that the $\beta$C1 protein contained in the betasatellite genome can substitute functionally for the CMG movement proteins (BV1 and BC1), which are regularly encoded by the DNA-B component, as demonstrated recently for tomato leaf curl New Delhi virus and ACMV with CLCuD betasatellite (Blawid et al., 2008; Saeed et al., 2007). Both the nuclear and the peripheral distribution of the $\beta$C1 protein encoded by the betasatellite are consistent with the hypothesis that it has a role in transporting the helper virus genome from the site of replication in the nucleus to the plasmodesmata for cell-to-cell movement (Saeed et al., 2007). In addition to symptom production in the presence of betasatellites, the infected plants showed the characteristic stem-curling and vein-swelling phenotypes for the CMGs trans-replicating the betasatellites, which could be due to a direct effect of the $\beta$C1 protein on the developmental microRNAs (miR165/166; Cui et al., 2004, 2005; Qazi et al., 2007; Saed et al., 2005; Yang et al., 2008). The accumulation of betasatellite in the absence of the DNA-B component was generally increased, in contrast to betasatellite accumulation in the presence of the DNA-B component, resulting in the enhancement of the characteristic stem-curling and vein-swelling phenotypes. There was an increase in defective forms of the DNA-B component in the presence of betasatellites (data not shown), possibly indicating a reduction in the functional role of the DNA-B component (Patil et al., 2007).

The recovery of the disease symptoms, which is the characteristic feature of the two virus species ACMV and EACMKV, was delayed when co-inoculated with betasatellites and, in most cases, they did not recover from the infection. The present studies also demonstrated that the two recently identified CMGs, EACMZV and EACMKV, trans-replicate betasatellites, accumulate the alphasatellite AYVA-1 and, respectively, show either symptom enhancement or suppression when co-infected (Bull et al., 2006; Maruthi et al., 2004). In contrast, EACMCV and all strain representatives of the EACMV species, namely EACMV-K24, EACMV-K201 and the EACMV-Ug isolates, -Ug2A and -K282, could neither trans-replicate betasatellites nor show any manifestation of symptom phenotype. When considering the different types of satellite interaction, the CMGs could be grouped into two different categories: those that interact with DNA satellites and those that do not (Table 1).

In contrast to the requirement of specific iteron sequences for trans-replication of DNA-B by the Rep protein encoded by the DNA-A component, the DNA satellites apparently do not require these molecular motifs for recognition by the Rep protein. The betasatellites are trans-replicated by a diverse range of begomoviruses, due to more relaxed recognition of the origin of replication (Arguello-Astorga et al., 1994; Fontes et al., 1992; Gladfelter et al., 1997;
Hanley-Bowdoin et al., 2000; Mansoor et al., 2003; Saunders et al., 2008). Analysis of the sequence of satellites and their helper viruses led to the prediction of a putative Rep-binding site in the AYVV sequence in the satellite conserved region (SCR; an approx. 200 nt region of high sequence conservation) that consists of an inverted repeat and multiple copies of the sequence GTCTCC and its complement GGAGAC (Idris et al., 2005; Saunders et al., 2008). However, until now, there has been no explanation of why some betasatellites are recognized by the Rep proteins of only some begomoviruses and not others (Eini et al., 2009). Recent studies showed that the cotton leaf curl Multan betasatellite (CLCuMuB) could adapt to the NW geminivirus CabLCuV after several generations of...

![Phylogenetic tree of Rep amino acid sequences](image)

Fig. 5. (a) Phylogenetic tree of Rep amino acid sequences of selected cassava-infecting geminiviruses used in this study. The tree is rooted on the predicted sequence of the corresponding protein of CabLCuV (a distantly related begomovirus originating from the NW). Bootstrap confidence values (1000 replicates) are shown. The scale at the base of the diagram is pairwise distance expressed as amino acid percentage substitution. (b) Sequence alignment of amino acid sequences of CMGs, showing conserved amino acids across the CMGs that potentially interact with betasatellites. The boxed region of Rep (nt 101–134) is the most prominent across the CMGs interacting with betasatellites.
inoculation in *N. benthamiana* through mutations in the region between the A-rich region and the SCR of CLCuMuB (Nawaz-ul-Rehman et al., 2009). Interestingly, the CMGs demonstrated differential interaction with the betasatellites, indicating the presence of significant diversity within the Rep sequences of CMG isolates and thus in their ability to bind the SCR of betasatellites in order to *trans*-replicate them. Phylogenetic analysis of the Rep proteins of all CMGs showed that the CMGs interacting with betasatellites clustered separately from the CMGs (EACMV and EACMCV) not interacting with betasatellites (Fig. 5a). Sequence analysis indicated the presence of several conserved residues in the N terminus of Rep proteins, of which region nt 101–134 is the most prominent (Fig. 5b) across the CMGs interacting with the betasatellites (and lacking in the non-interacting CMGs) (Campos-Olivas et al., 2002; Vega-Rocha et al., 2007). However, mutational studies of these residues or gene-swapping experiments for the corresponding sequences could help in identification of domains responsible for the differential interaction with betasatellites (Fig. 5b). The interaction of CMGs with betasatellites was conserved across the different betasatellite species (AYVB, EpYMVB and HYVB) studied in this experiment, which was in contrast to differential interaction of the begomoviruses CLCuMV and HYVV with different betasatellites (Saunders et al., 2008). Irrespective of the geographical origin of the begomovirus betasatellites (AYVV from Singapore, EpYMV from Japan and HYVV from the UK), they showed conserved interaction with all of the interacting CMGs. This indicates that the ability of the CMGs to interact differentially with betasatellites was conserved across the geographical region or plant species with which they were associated.

The nanovirus-like components cannot strictly be classified as DNA satellites because of their auto-replication capacity (Kon et al., 2009); thus, they have been called satellite-like molecules. These nanovirus-like ssDNAs are usually approximately 1375 nt in size and, in addition to the Rep proteins, of which region nt 101–134 is the most prominent (Fig. 5b) across the CMGs interacting with the betasatellites (and lacking in the non-interacting CMGs) (Campos-Olivas et al., 2002; Vega-Rocha et al., 2007). However, mutational studies of these residues or gene-swapping experiments for the corresponding sequences could help in identification of domains responsible for the differential interaction with betasatellites (Fig. 5b). The interaction of CMGs with betasatellites was conserved across the different betasatellite species (AYVB, EpYMVB and HYVB) studied in this experiment, which was in contrast to differential interaction of the begomoviruses CLCuMV and HYVV with different betasatellites (Saunders et al., 2008). Irrespective of the geographical origin of the begomovirus betasatellites (AYVV from Singapore, EpYMV from Japan and HYVV from the UK), they showed conserved interaction with all of the interacting CMGs. This indicates that the ability of the CMGs to interact differentially with betasatellites was conserved across the geographical region or plant species with which they were associated.

Production of agro-infectious clones of CMGs. Clones have been made from a number of virus isolates representative of different CMG species: ACMV, EACMV, EACMCV, EACMKV and EACMZV. Most of the EACMV-like begomovirus clones (EACMV-KE[KE: Kat: K24:01], EACMV-KE[KE: Msa: K201:02], EACMV-UG[KE: Wot: K282:02], EACMVK[KE: Keh: K229:02], EACMZV[KE: Kib: K275:02] and EACMZV[KE: Fel: K19:01]) used to produce the partial tandem dimers were the generous gift of Dr John Stanley, John Innes Centre, Norwich, UK, and were obtained as monomers in Invitrogen’s TOPO cloning vector (Bull et al., 2006). To simplify the nomenclature, these viruses will be referred to as EACMV-K24, EACMV-K201, EACMV-K282, EACMVK-K229, EACMZV-K275 and EACMZV-K19. First, the approximately 1.0 kb partial repeat of DNA-A and DNA-B components, encompassing the intergenic region, was cloned into the plbuescript II SK (+) cloning vector (Strategene). Second, the full-length viral DNA was cloned as a tandem repeat into the SalI restriction site for DNA-A and the Sce site for DNA-B in plbuescript II SK (+) with partial viral DNA. The newly constructed infectious clones were tested for infectivity by particle bombardment into both *N. benthamiana* and cassava (cv. 60444) in order to confirm their identity and integrity. Further, the tandem-repeated partial dimers of the above CMG DNA-A and DNA-B components were spliced from the cloning vector plbuescript II SK (+) with BssHII and subcloned into the compatible Aic site of a modified version of the binary vector pBin ¹, referred to as AKK1420 (Collier et al., 2005; van Engelen et al., 1995). Similarly, previously studied, tandem repeat infectious clones of
EACMV-UG[Ug: Svr2: 97], namely DNA-A (GenBank accession no. AF126806), EACMV-UG[Ug: Svr3: 97] DNA-B (AF126807), EACMCV-CM[CI: 98] (AF259896 and AF259897) and CabLcuV-[US: Flo: 96] DNA-A (U65529) and DNA-B (U65530) components, were also subcoloned into the binary vector AKK1420 (Fondong et al., 2000; Pita et al., 2001; Turnage et al., 2002) for agro-inoculation studies. Other agro-infectious geminiviruses and the CMG clones were used in this study and were ICMV-IN[NI: Mah: 88] (ICMV; GenBank accession nos AI314739 and AI314740), SLCMV-LK[LC: Col: 98] (SLCMV; AI314737 and AI314738) (Saunders et al., 2002), ACMV-[KE: 844: 82] (ACMV; J02057 and J02058) (Klinkenberg et al., 1989), SACTMV-ZA (SACTMV; AI55806 and AI55807) (Berrie et al., 1998), TGMV-[BR: Com: 84] (K02029 and K02030) (von Arnim & Stanley, 1992), BCTV-A [US: Cal: 85] (X04144) (Stanley et al., 1986) and BeYDV-ZA [Za: Mpu: 94] (BeYDV; Y11023) (Liu et al., 1997).

The agro-infectious clones of three geminivirus betasatellites used in this study, which are the Ageratum yellow vein betasatellite from Singapore (AYVB-[SG: 92]; GenBank accession no. AI252072) (Saunders et al., 2002), HYVB from the UK (HYVB-[UK: Nor: 2: 99]; AI543430) and EpMYVB (EpYVB-[JR: MNS2: 00]; AI38938) (Saunders et al., 2003), were obtained from Dr John Stanley (John Innes Centre, Norwich, UK).

The alphasatellites used in this study were the two isolates of the Ageratum yellow vein alphasatellite (AYVA) from Singapore (AYVA-[SG: 98]): AYVA-1 (GenBank accession no. AJ238493) (Saunders & Stanley, 1999) and AYVA-2 (AJ416153) (Saunders et al., 2002), which were also the gifts of Dr Stanley.

Plasmids of all the agro-infectious clones were transformed into competent cells of Agrobacterium strain GV3101 by electroporation (Bio-Rad Gene Pulser, 0.2 cm cuvettes, 25 μF, >2.5 kV).

**Infectivity studies by agro-infiltration.** The infectious geminivirus clones in Agrobacterium (GV3101) were cultured in Luria–Bertani broth, with the antibiotics kanamycin (25 μg ml⁻¹) and rifampicin (10 μg ml⁻¹) for selection, in 28 °C at 200 r.p.m. in a shaking incubator for 24–30 h to an OD₆₀₀ of 1.0. The culture was centrifuged before the pellet was resuspended in infiltration medium with 10 mM MgCl₂, 1/4-strength MS salts, 10 mM MES and 150 μM acetosyringone and incubated for approximately 3 h at room temperature (Wydro et al., 2006). Equal volumes of the cultures of each component to be inoculated were mixed just before infiltration and infiltrated under the leaf surface of the top three, fully opened leaves of 3-week-old N. benthamiana using a 1 ml norm-ject syringe without the needle (Henke-Sass, Wolf GmbH).

**Symptom scoring.** The inoculated plants were maintained in controlled growth chambers with a constant temperature of 28 °C, 70% relative humidity and 200 μE m⁻² s⁻¹ light with an 16 h light and 8 h dark period. All experiments were repeated at least four times in N. benthamiana, with the symptom progression recorded every alternate day for a period of 1 month, beginning from the day when symptoms first appeared. Symptom score was done using a scale of 0–5 (0 indicates absence of symptoms and 5 indicates the necrosis/death of the plant; Faquet & Fargette, 1990). The symptomatic plants were photographed using a Nikon D80 digital SLR camera and the images were processed for clarity using Adobe Photoshop CS2.

**PCR analyses and Southern hybridization.** PCR analysis was done for randomly selected sample plants for all combinations of virus inoculations, with primers designed to specifically amplify selected regions of DNA-A, DNA-B, alphasatellites or betasatellites. Plant genomic DNA was extracted at 1 and 3 weeks p.i. from a fully opened third leaf from the top of the plant. Genomic DNA extractions were done using a Qiagen DNeasy DNA extraction kit. The DNA was quantified by spectrophotometry (Bio-Rad Smart Spec 3000 UV spectrophotometer). Genomic DNA (4 μg) was fractionated in 1% agarose gel and transferred for Southern hybridization using standard methods (Sambrook & Russell, 2001). Southern hybridizations were performed using a DIG High Prime DNA labelling and detection kit (Roche Applied Science) and, for specific detection of each component, AV1 (DNA-A), BC1 (DNA-B), JC1 (AYYB) and Rep (AYV-1 or AYV-2) were used as probes. Autoradiography was done using Amersham high-performance chemiluminescence film (GE Healthcare). The exposed films were developed in an automated developer (Kodak X-Omat) and the autoradiograms were scanned and adjusted for clarity in Adobe Photoshop CS2. The virus titres were estimated by quantifying the ssDNA forms on the autoradiogram by using the software IMAGE-J.

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**REFERENCES**


Interaction between cassava geminivirus and satellites


