

# Infectivity, pseudorecombination and mutagenesis of Kenyan cassava mosaic begomoviruses

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Cloned DNA-A and DNA-B components of Kenyan isolates of East African cassava mosaic virus (EACMV, EACMV-UG and EACMV-KE2), East African cassava mosaic Kenya virus (EACMKV) and East African cassava mosaic Zanzibar virus (EACMZV) are shown to be infectious in cassava. EACMV and EACMKV genomic components have the same iteron sequence (GGGGG) and can form viable pseudorecombinants, while EACMZV components have a different sequence (GGAGA) and are incompatible with EACMV and EACMKV. Mutagenesis of EACMZV has demonstrated that open reading frames (ORFs) AV1 (encoding the coat protein), AV2 and AC4 are not essential for a symptomatic infection of cassava, although mutants of both ORF AV1 and AV2 produce attenuated symptoms in this host. Furthermore, ORF AV1 and AV2 mutants were compromised for coat protein production, suggesting a close structural and/or functional relationship between these coding regions or their protein products.

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## INTRODUCTION

Begomoviruses (family *Geminiviridae*, genus *Begomovirus*) associated with cassava mosaic disease (CMD) are found throughout sub-Saharan Africa where cassava [*Manihot esculenta* (Crantz)] is the primary food crop. They are transmitted by the whitefly *Bemisia tabaci* (Gennadius) and are considered to be one of the most damaging vector-borne pathogens of any African crop, with estimated losses in excess of US\$1.5 billion a year (Thresh *et al.*, 1994). Several distinct species are associated with CMD, namely *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Kenya virus* (EACMKV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV) and *South African cassava mosaic virus* (SACMV) in Africa, and *Sri Lankan cassava mosaic virus* (SLCMV) and *Indian cassava mosaic virus* (ICMV) in the Indian subcontinent (Stanley *et al.*, 2005).

All of these begomoviruses have bipartite genomes comprising a DNA-A component required for replication and encapsidation and a DNA-B component required for virus movement (Hanley-Bowdoin *et al.*, 1999). Both components have a highly conserved intergenic common region (CR) containing a stem-loop structure with an invariant nonanucleotide motif (TAATATTAC) and bidirectional, partially overlapping open reading frames (ORFs) AV1 and AV2 on the virion-sense strand and AC1–AC4 on the complementary-sense strand that are typical of Old World bipartite begomoviruses. The ACMV coat protein (CP), encoded by ORF AV1 (Townsend *et al.*, 1985), is principally involved in the encapsidation of the DNA components for transmission by the whitefly vector (Briddon *et al.*, 1990; Liu *et al.*, 1998). It has also been implicated in subcellular targeting (Unselde *et al.*, 2001, 2004), but is not essential for systemic infection in *Nicotiana benthamiana* (Stanley & Townsend, 1986; Ward *et al.*, 1988), although the extent of CP involvement in virus systemic movement may depend on virus–host adaptation (Pooma *et al.*, 1996). Additionally, ACMV CP mutants characteristically accumulate only low levels of single-stranded (ss) DNA (Stanley & Townsend, 1986). The function of the upstream, overlapping ORF AV2 product is uncertain. ACMV ORF AV2 mutants remain infectious in *N. benthamiana* (Etessami *et al.*, 1989) and those of tomato leaf curl New Delhi virus (ToLCNDV) produced attenuated symptoms and accumulated low levels of viral DNA (Padidam *et al.*, 1996). Similarly, the function of ORF AC4

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Sequences of primers used are available as supplementary material in JGV Online.

is not clear, since disruption of the coding sequence in ACMV had no effect on the phenotype in *N. benthamiana* (Eteessami *et al.*, 1991). However, there is compelling evidence to suggest that ACMV AC4 may counter a plant defence mechanism initiated by expression of the replication-associated protein (Rep) that would otherwise lead to severe necrosis and plant death (van Wezel *et al.*, 2002). In addition, ACMV AC4 has been shown to be an RNA-silencing suppressor protein that binds to small interfering and micro RNAs and induces developmental abnormalities in transgenic plants (Chellappan *et al.*, 2005). This suggests a role in cell-cycle control, which has been proposed for the beet curly top virus (BCTV) C4 positional homologue (Latham *et al.*, 1997). The fact that the AC4 proteins of EACMCV and ICMV do not show similar silencing suppression activity (Vanitharani *et al.*, 2004) may reflect the considerable diversity observed within AC4 sequences of distinct begomovirus species (Bull *et al.*, 2006).

Infectious clones of ACMV were produced from diseased cassava over two decades ago (Stanley, 1983; Stanley & Gay, 1983) and remain the only examples to originate from Kenya. Although ACMV DNA-A is capable of limited systemic spread in *N. benthamiana* in the absence of DNA-B (Klinkenberg & Stanley, 1990), both components are required for a systemic symptomatic infection. The clones induced a severe downward leaf-curling phenotype and stunted growth when mechanically inoculated to *N. benthamiana*. However, despite replicating in cassava leaf discs (Zhang & Gruissem, 2003), the cloned ACMV components failed to systemically infect cassava as a consequence of defects in the CP and DNA-B component (Briddon *et al.*, 1998; Liu *et al.*, 1998). Since these studies, only a few other clones have been successfully inoculated to cassava, namely an ACMV isolate from Nigeria (Briddon *et al.*, 1998), SACMV (Berrie *et al.*, 2001), SLCMV (Saunders *et al.*, 2002) and ICMV (Rothenstein *et al.*, 2005).

Pseudorecombination occurs during mixed infections in the field and provides a means for the generation of new viruses by the exchange of genomic components. For example, Pita *et al.* (2001) showed that EACMV-UG2 DNA-A is capable of *trans*-replicating EACMV-UG3 DNA-B, representing the first demonstration of infectivity of EACMV clones to cassava. The resulting symptoms were particularly severe and this pseudorecombinant has dominated the initially identified EACMV-UG1 isolate in Uganda (Zhou *et al.*, 1997). Synergistic interactions also arise between viruses, as seen in *N. benthamiana* plants co-inoculated with Cameroon isolates of ACMV and EACMV that showed more severe symptoms than in plants infected with either virus alone (Fondong *et al.*, 2000), as well as between EACMV-UG2 and ACMV-[UG] (Harrison *et al.*, 1997; Pita *et al.*, 2001) and in natural infections between EACMV and ACMV from Nigeria (Ogbe *et al.*, 2003).

Frischmuth *et al.* (1993) showed that pseudorecombination compatibility between ACMV and ICMV components is

restricted by *trans*-replication rather than an inability to spread throughout the plant. Rep-binding motifs, referred to as iterons (Argüello-Astorga *et al.*, 1994) and located in the CR 5' of the nonanucleotide motif, are crucial in determining the compatibility of genomic components for *trans*-replication. The interaction between Rep and the iteron is highly specific, usually preventing any functional interaction between components of distinct begomovirus species (Fontes *et al.*, 1992, 1994a; Orozco *et al.*, 1998; Chatterji *et al.*, 2000). However, this incompatibility can be overcome by exchange of intergenic region sequences, which has been shown to occur frequently between begomoviruses and their associated components, both experimentally (Roberts & Stanley, 1994; Saunders *et al.*, 2001) and in the field (Saunders *et al.*, 2002).

We have recently undertaken a comprehensive investigation of the epidemiology of begomoviruses associated with CMD in Kenya (Bull *et al.*, 2006). During the course of this study, we cloned numerous full-length genomic components of EACMV and its distinct strains EACMV-UG and EACMV-KE2, as well as EACMKV and EACMZV. Here, we demonstrate the biological activity of selected clones and investigate their compatibility in pseudorecombination experiments using both the experimental host *N. benthamiana* and the natural host cassava. Finally, we have undertaken a mutational analysis on ORFs AV1, AV2 and AC4 to investigate their contribution to CMD.

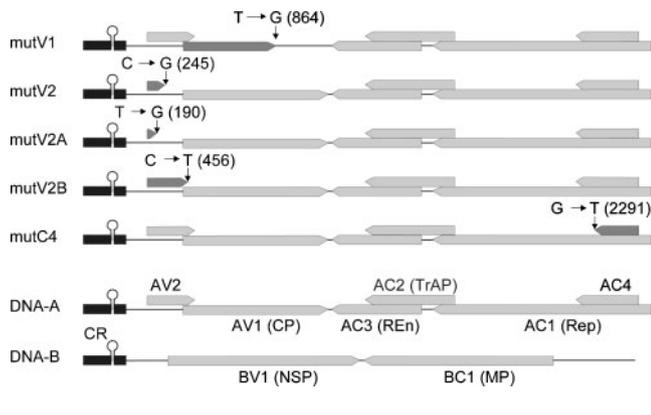
## METHODS

**Plant inoculation and maintenance.** *N. benthamiana* and tissue-cultured cassava 'Ebwanateraka' (kindly provided by M. N. Maruthi, Natural Resources Institute, Medway, UK) were biolistically inoculated with cloned genomic components as described by Briddon *et al.* (1998). The plants were maintained in insect-free glasshouses at the JIC at 25–30 °C and with supplementary lighting to give a 16 h photoperiod. Symptoms of CMD in cassava were classified by visually assessing the plant and designating a score between 0 (no symptoms) and 5 (severe symptoms). All viruses were maintained and manipulated under DEFRA licence PHL 185A/4538 (7/2003).

**Plasmid constructs.** Full-length DNA-A and DNA-B components of the Kenyan begomoviruses EACMZV-[K18], EACMV-[K24], EACMV-KE2[K48], EACMKV-[K261] and EACMV-UG[K282] (Bull *et al.*, 2006) were used in this analysis.

**Site-directed mutagenesis.** Point mutations were introduced into AV1, AV2 and AC4 coding sequences of the cloned EACMZV-[K18] DNA-A component using a QuikChange site-directed mutagenesis kit (Stratagene) and the overlapping primers shown in Supplementary Table S1 (available in JGV Online). Nonsense codons were introduced to replace codons encoding tyrosine<sup>177</sup> in ORF AV1 (mutV1), tyrosine<sup>24</sup> (mutV2), leucine<sup>6</sup> (mutV2A) and glutamine<sup>95</sup> (mutV2B) in ORF AV2 and serine<sup>66</sup> in ORF AC4 (mutC4) (Fig. 1).

**Detection of viral DNA.** DNA was extracted from newly emerging and/or symptomatic leaves approximately 15 days post-inoculation (days p.i.) using a Nucleon Phytopure plant DNA extraction kit (Amersham Biosciences). Samples (10 µg) were fractionated on 1% agarose gels in TNE buffer [40 mM Tris/HCl (pH 7.5), 200 mM sodium acetate, 20 mM EDTA], transferred to Hybond-NX membranes



**Fig. 1.** Maps of EACMZV DNA-A and DNA-B components. For simplicity, the circular genomic components are depicted as linear maps. The position and orientation of ORFs (light grey) and mutated ORFs (dark grey) are shown relative to the common region (black box). The mutations introduced into ORFs AV1, AV2 and AC4 are indicated. CP, Coat protein; TrAP, transcriptional activator protein; REEn, replication enhancer protein; Rep, replication-associated protein; NSP, nuclear shuttle protein; MP, movement protein.

(Amersham Biosciences) and hybridized to [ $\alpha$ - $^{32}$ P]dCTP-labelled EACMV DNA-A or DNA-B probes produced using a Random Primer DNA labelling kit (Gibco-BRL) and a NucTrap probe purification column (Stratagene).

A PCR-based approach to isolate full-length DNA-A and DNA-B components for nucleotide sequencing has been described by Bull *et al.* (2006).

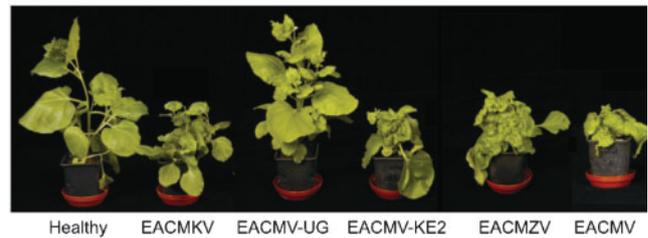
**Analysis of CP expression.** Proteins were extracted from *N. benthamiana* and cassava as described by von Arnim *et al.* (1993) and fractionated on 12% polyacrylamide gels (Laemmli, 1970) before transfer to nitrocellulose membrane (Whatman - Schleicher & Schuell) using a semi-dry transfer cell (Bio-Rad). CP was detected by immunolabelling using polyclonal antiserum raised against purified ACMV (Stanley & Townsend, 1986).

## RESULTS

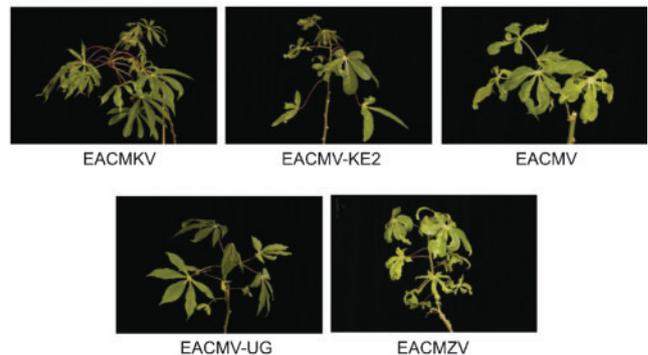
### Infectivity of cognate DNA-A and DNA-B components

Cloned components of EACMV, EACMV-UG, EACMV-KE2, EACMKV and EACMZV all induced downward leaf curling and stunted growth in *N. benthamiana* (Fig. 2). Infection by EACMZV was slightly more aggressive than either EACMV or EACMV-KE2, whereas EACMV-UG caused only mild curling and slight stunting. *N. benthamiana* plants infected with EACMKV developed an intermediate phenotype. Disease symptoms became apparent between 9 and 10 days p.i. for the more aggressive isolates (EACMZV, EACMV and EACMV-KE2) and between 11 and 15 days p.i. for EACMKV and EACMV-UG (Table 1). Southern blot hybridization confirmed the presence of

### *Nicotiana benthamiana*



### Cassava



**Fig. 2.** Symptoms induced in *N. benthamiana* and cassava by Kenyan begomoviruses.

both virus components in systemically infected tissues (data not shown).

The cloned components of the representative viruses were also infectious in cassava, inducing typical disease symptoms (yellow mosaic and leaf distortion) between 10 and 15 days p.i. (Table 1). Symptom severity was overall commensurate with that seen in *N. benthamiana*, with EACMV-UG giving a markedly mild phenotype and EACMZV, EACMV, EACMV-KE2 and EACMKV inducing severe symptoms (Fig. 2) that continued to be expressed in new growth following ratooning, reflecting the aggressive nature of these virus clones in cassava. ACMV-infected cassava plants frequently recover (symptoms reducing in severity or disappearing entirely) due to post-transcriptional gene silencing (PTGS), while plants infected with the more aggressive EACMCV do not recover and cannot silence it by PTGS (Chellappan *et al.*, 2004). The regrowth following ratooning frequently shows a change in symptom severity for viruses exhibiting the recovery phenotype. The lack of this suggests that EACMZV, EACMV, EACMV-KE2 and EACMKV are viruses of the aggressive type that can overcome PTGS. In all cases, symptom severity associated with the cloned components resembled the phenotype associated with the field isolates from which the viruses were isolated.

### Infectivity of pseudorecombinants

Pseudorecombinants produced by exchanging components of EACMV, EACMV-KE2, EACMKV and EACMV-UG all

**Table 1.** Infectivity of cognate DNA-A and DNA-B components of representative begomovirus species and strains sampled from districts throughout Kenya

Isolate	<i>N. benthamiana</i>		Cassava			
	Infected/inoculated	days p.i.	Infected/inoculated	days p.i.	Severity*	Field severity*
EACMZV-[K18]	12/16	10	2/2	10	5	5
EACMV-[K24]	18/20	9	2/2	11	5	4
EACMV-KE2[K48]	15/16	10	2/2	12	5	5
EACMV-UG[K282]	10/15	15	2/2	15	3	3
EACMKV-[K261]	16/20	11	2/2	12	4	3

\*Severity rating based on 0 (asymptomatic) to 5 (severe mosaic disease and leaf distortion).

gave a disease phenotype in *N. benthamiana* (Table 2). Plants infected with EACMV DNA-A gave a range of symptoms depending on the DNA-B component with which it was associated (Fig. 3). The most severe phenotype was produced with EACMV-KE2 DNA-B, the least severe with EACMV-UG DNA-B and EACMKV DNA-B gave an intermediate phenotype. Similarly, plants infected with EACMV-KE2 DNA-A produced severe symptoms with EACMV DNA-B, intermediate symptoms with EACMKV DNA-B and least severe symptoms with EACMV-UG DNA-B. However, symptoms in *N. benthamiana* induced by either EACMKV or EACMV-UG DNA-A components with any other DNA-B component were largely indistinguishable, comprising mild stunting and leaf curling. In contrast, all pseudorecombinants that contained either the DNA-A or DNA-B component of EACMZV failed to give symptoms in *N. benthamiana*. However, a low level of EACMZV DNA-A was detected by Southern blotting when

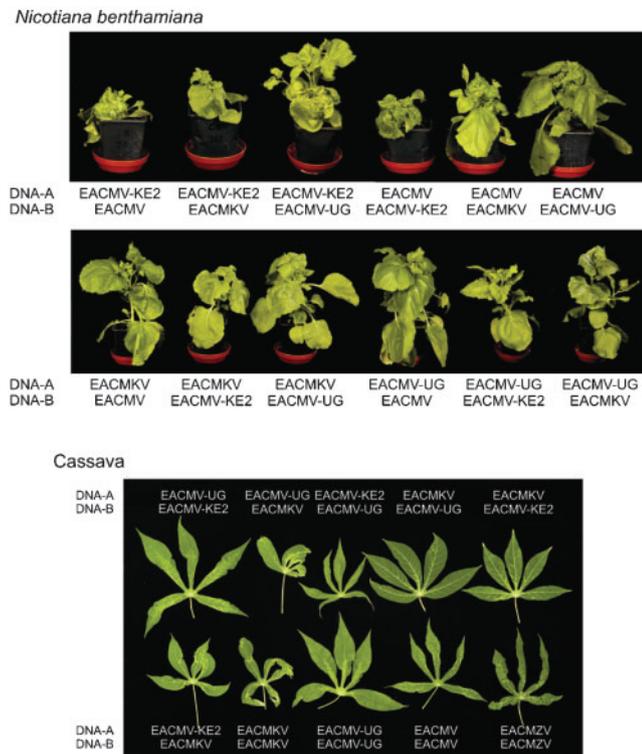
this component was co-inoculated with either EACMV, EACMV-KE2 or EACMV-UG DNA-B. PCR-mediated isolation, cloning and sequencing of a full-length DNA-A component confirmed its presence in the asymptomatic tissues. Furthermore, Southern blotting and PCR amplification using component-specific primers (Bull *et al.*, 2006) failed to detect DNA-B components in newly emerging leaves. Subsequent Southern blot analysis of 18 asymptomatic *N. benthamiana* plants inoculated with EACMZV DNA-A alone showed that the component was present at low levels in newly emerging leaves of three plants by 20 days p.i. (data not shown) and its integrity was confirmed by PCR amplification of the full-length component and sequence analysis. Our finding that EACMZV DNA-A can spread systemically in *N. benthamiana* following biolistic delivery is consistent with an earlier observation using agroinoculation of ACMV DNA-A (Klinkenberg & Stanley, 1990).

**Table 2.** Infectivity of pseudorecombinants

Infectivity is given as the number of plants infected/number inoculated. Symptom abbreviations: mild leaf curl (mlc); severe leaf curl (slc); mild stunting (mst); severe stunting (sst); mosaic (m). ND, Not done; Nb, *N. benthamiana*.

DNA-B component	DNA-A component									
	EACMZV		EACMV		EACMV-KE2		EACMKV		EACMV-UG	
	Nb	cassava	Nb	cassava	Nb	cassava	Nb	cassava	Nb	cassava
EACMZV	12/14	2/2	0/20	ND	0/32	ND	0/20	ND	0/32	ND
	sst/slc	m								
EACMV	0/10*	ND	8/10	2/2	8/10	2/2	9/9	2/2	8/10	2/2
			sst/slc	m	slc/mst	m	mst/mlc	m	mst/mlc	m
EACMV-KE2	0/32*	ND	9/10	2/2	12/14	2/2	9/10	2/2	15/20	2/2
			slc/mst	m	sst/slc	m	mst/mlc	m	mst/mlc	m
EACMKV	0/20	ND	8/10	2/2	10/10	2/2	6/6	2/2	5/10	2/2
			slc/mst	m	mst/mlc	m	mst/mlc	m	mst/mlc	m
EACMV-UG	0/32*	ND	9/9	2/2	19/20	2/2	9/10	2/2	7/14	2/2
			slc/mst	m	mst/mlc	m	mst/mlc	m	mst/mlc	m

\*Asymptomatic plants show limited systemic movement of DNA-A but not DNA-B.



**Fig. 3.** Symptoms induced in *N. benthamiana* and cassava by pseudorecombinants produced by exchanging DNA-A and DNA-B components of Kenyan begomoviruses.

The pseudorecombinants that were infectious in *N. benthamiana* also induced mosaic disease symptoms in cassava, although they were generally less severe than those induced by DNA-A and DNA-B components derived from the same species or strain (Fig. 3; Table 2). EACMV-UG, shown to be the least virulent of the viruses in both *N. benthamiana* and cassava, also gave relatively mild symptoms in cassava as a component of a pseudorecombinant. Other pseudorecombinants produced very similar phenotypes that often varied throughout the plant, with some shoots showing signs of recovery and others displaying a yellow mosaic. Furthermore, the symptoms sometimes increased in severity as the plant aged. This was more likely to occur in plants infected with pseudorecombinants that initially gave a more severe mosaic phenotype (for example, EACMV-KE2 DNA-A and EACMKV DNA-B), whereas other pseudorecombinants (for example, EACMKV DNA-A and EACMV-UG DNA-B) were likely to show signs of recovery.

### Mutation of ORF AV1

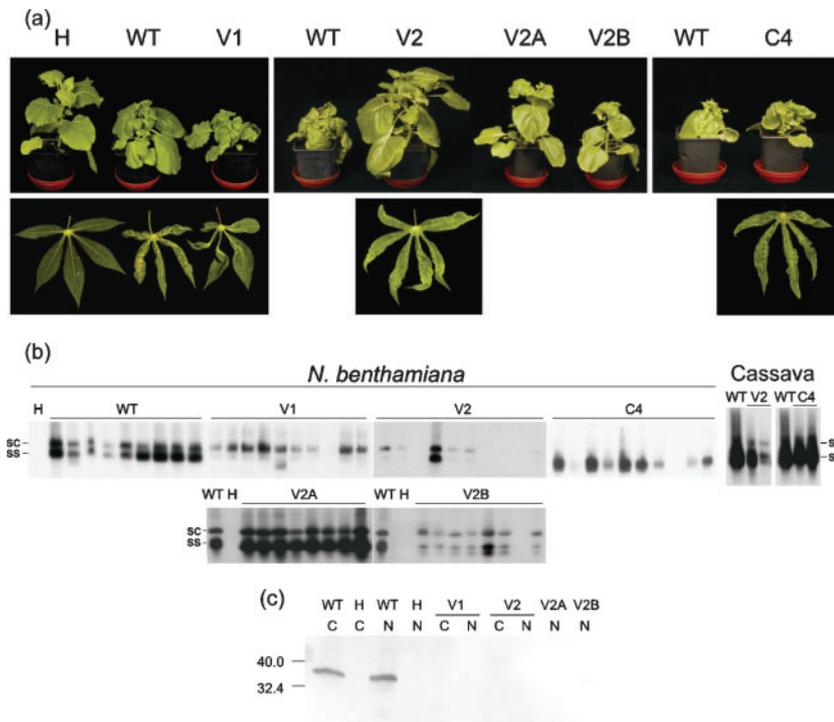
The EACMZV CP coding sequence (257 aa) was disrupted by replacing tyrosine<sup>177</sup> with an in-frame nonsense codon in ORF AV1, downstream of the overlapping ORF AV2 (mutant mutV1; Fig. 1). All ten *N. benthamiana* plants co-inoculated with mutV1 and EACMZV DNA-B exhibited

severe downward leaf curling and stunted growth by 12 days p.i., comparable to the wild-type infection (Fig. 4a). In addition, two inoculated cassava plants displayed a mosaic phenotype and leaf distortion, also at approximately 12 days p.i., although symptoms were slightly milder than in plants infected with the wild-type virus. Southern blot analysis revealed a marked reduction in the level of ssDNA in *N. benthamiana* (Fig. 4b) as well as in cassava when compared with the wild-type infection. Sequence analysis of three full-length DNA-A clones isolated from two cassava plants and one *N. benthamiana* plant confirmed that the mutation had been retained *in vivo* and that no other nucleotide changes had occurred. Western blot analysis showed that CP was expressed in *N. benthamiana* and cassava plants infected with wild-type virus, but not with mutV1 (Fig. 4c), confirming that the mutation had disrupted CP expression.

### Mutation of ORF AV2

The EACMZV AV2 coding sequence (118 aa) was initially disrupted by replacing tyrosine<sup>24</sup> with an in-frame nonsense codon in ORF AV2, upstream of the overlapping ORF AV1 (mutant mutV2; Fig. 1). Four out of ten *N. benthamiana* plants inoculated with mutV2 and EACMZV DNA-B expressed symptoms by 12 days p.i., which were significantly milder than in plants infected with the wild-type virus (Fig. 4a). Plants also showed some variation in phenotype, ranging from slight downward leaf curling to moderate stunting of growth. Two cassava plants inoculated with mutV2 displayed mosaic disease symptoms that were milder than in plants infected with the wild-type virus. Southern blot analysis showed variable and generally low levels of viral DNA accumulation in *N. benthamiana* and cassava, although both ssDNA and double-stranded DNA (dsDNA) forms accumulated in cassava (Fig. 4b). Sequence analysis of a full-length DNA-A clone isolated from a *N. benthamiana* plant exhibiting mild leaf curling (clone piv2p15) confirmed that the mutation had been retained *in vivo* and that no other nucleotide changes had occurred. However, a clone isolated from a plant exhibiting mild leaf curling and stunting (clone piv2p11) retained the introduced mutation, but also had a C to T transition at position 2681 in the CR. Furthermore, a clone isolated from a plant exhibiting slightly more severe symptoms (clone piv2) had a T to G transversion at position 2732 in the CR and an A to G transition at position 2372 in the overlapping AC1/AC4 ORFs. The latter did not affect the Rep sequence, but caused a phenylalanine<sup>39</sup> to serine substitution in AC4. The plant exhibiting a relatively high level of viral DNA accumulation (Fig. 4b) was not analysed and consequently we cannot rule out the possibility that the mutation had reverted on this occasion. A full-length DNA-A clone isolated from cassava showed no alterations other than the introduced mutation in ORF AV2.

To investigate the phenotypes of the mutants produced *in vivo* and to ensure that they represent biologically active



**Fig. 4.** (a) Infectivity of EACMZV mutants in *N. benthamiana* and cassava. Plants were either healthy (H) or infected with wild-type (WT) virus or mutants mutV1 (V1), mutV2 (V2), mutV2A (V2A), mutV2B (V2B) and mutC4 (C4). (b) Southern blot analyses of DNA extracted from *N. benthamiana* and cassava plants, probed for EACMZV DNA-A. The positions of viral single-stranded (ss) and supercoiled (sc) DNA forms are indicated. (c) Western blot analysis of EACMZV coat protein expression in *N. benthamiana* (N) and cassava (C). Proteins were extracted from healthy plants or plants infected with wild-type virus and mutants mutV1, mutV2, mutV2A and mutV2B. The positions of size markers (kDa) are indicated.

components, DNA-A clones piv2p15, piv2p11 and piv2 were co-inoculated with EACMZV DNA-B to *N. benthamiana*. Clone piv2 produced leaf curling and stunting by 12 days p.i. which was initially more severe than the symptoms caused by piv2p11 and piv2p15, consistent with an influence of the additional mutations on phenotype, although symptoms induced by all three clones became indistinguishable by 20 days p.i.

Due to the overlapping nature of ORFs AV1 and AV2, the possibility that the mutation in mutV2 could affect CP expression was considered. To test this, protein extracts from infected *N. benthamiana* and cassava plants were analysed by Western blotting. Unexpectedly, mutV2-infected plants contained no detectable accumulation of CP (Fig. 4c), implying that the mutation indeed had a detrimental effect on CP expression. This problem was addressed by designing two additional ORF AV2 mutants in which in-frame nonsense mutations replaced either leucine<sup>6</sup> (mutV2A) or glutamine<sup>95</sup> (mutV2B), the latter mutation located within the overlapping virion-sense ORFs, although the CP coding sequence remained unaffected (Fig. 1). When co-inoculated with EACMZV DNA-B, both mutants induced leaf curling and stunted growth in *N. benthamiana* by 13 days p.i. Symptoms were more severe for mutV2B, although both mutants induced less severe symptoms than the wild-type virus (Fig. 4a). Viral DNA accumulation was unaffected by the mutation in mutV2A while the accumulation of mutV2B, particularly the ssDNA, was significantly reduced (Fig. 4b). Once again, Western blot analysis failed to detect CP in protein extracts from *N. benthamiana* plants infected with either of

these ORF AV2 mutants (Fig. 4c), indicating that both were compromised for CP production.

### Mutation of ORF AC4

The AC4 coding sequence (85 aa) was disrupted by replacing serine<sup>66</sup> with an in-frame nonsense codon (mutant mutC4; Fig. 1). The mutation did not alter the amino acid encoded by the overlapping ORF AC1. All ten *N. benthamiana* plants co-inoculated with mutC4 and EACMZV DNA-B developed severe wild-type symptoms by 12 days p.i. Furthermore, symptoms in two inoculated cassava plants were indistinguishable from those in plants infected with the wild-type virus (Fig. 4a). Southern blot analysis showed some variation in viral DNA accumulation in *N. benthamiana* plants, although mutC4 could accumulate to almost wild-type levels. MutC4 and wild-type virus accumulated to similar levels in cassava (Fig. 4b). Sequence analysis of full-length DNA-A components isolated from two *N. benthamiana* plants and two cassava plants showed that the mutation had been retained *in vivo* and that no other nucleotide changes had occurred.

### DISCUSSION

Using a biolistic delivery system, we have shown that cloned DNA-A and DNA-B genomic components of EACMV (including two distinct strains, EACMV-KE2 and EACMV-UG), EACMKV and EACMZV, representative of the begomovirus population associated with CMD in Kenya (Bull *et al.*, 2006), are infectious in cassava.



crucial role in Rep binding (Fontes *et al.*, 1994a), was identical between EACMV strains and EACMKV (GGGGG). In addition, this core sequence occurs as either an exact or partial repeat in both iteron 1 and the 5' core sequence of iteron 2, and as an inverted repeat in iteron 3. This high level of conservation explains the compatibility of their components for replication. However, the 3' core sequence of EACMZV (GGAGA) differs from those of EACMV and EACMKV, and a 5' core sequence does not occur in EACMZV, suggesting why EACMZV was unable to form viable pseudorecombinants with these viruses. Indeed, the arrangement of EACMZV iterons is more similar to isolates of ACMV that also have the core sequence GGAGA. Despite this, pseudorecombinants constructed between EACMZV and ACMV were not infectious in *N. benthamiana* (data not shown), indicating that factors other than a conserved iteron core sequence are necessary for *trans*-replication compatibility (Fontes *et al.*, 1994b).

We have investigated the contribution of CP, AV2 and AC4 proteins to the disease phenotype in cassava using EACMZV DNA-A mutants. The results indicate that EACMZV CP expression is not essential for symptomatic infection of cassava, although the CP mutant accumulated only low levels of ssDNA and produced slightly attenuated symptoms. Similarly, a reduction in ssDNA accumulation has been reported for ACMV CP mutants in *N. benthamiana* (Stanley & Townsend, 1986; Eteessami *et al.*, 1989) as well as for isolates of bean golden yellow mosaic virus, ToLCNDV and tomato yellow leaf curl virus (Azzam *et al.*, 1994; Padidam *et al.*, 1995, 1996; Wartig *et al.*, 1997). This may be attributable to a reduction of ssDNA being sequestered into virions in the absence of CP expression. Although ACMV CP has been shown to facilitate nuclear import/export and transport of the virus to the cell periphery (Unselde *et al.*, 2001), it is likely that the ORF AV1 mutant remains viable due to functional redundancy between CP and DNA-B-encoded BV1 (Pooma *et al.*, 1996).

Previous reports have demonstrated that ACMV ORF AV2 is not essential for infection in *N. benthamiana* (Ward *et al.*, 1988; Eteessami *et al.*, 1989). Here, we have shown that EACMZV ORF AV2 mutants are infectious in *N. benthamiana* and cassava, although symptoms in both hosts were attenuated and levels of viral DNA accumulation were reduced. Mild symptoms and a reduction in the level of viral DNA were also associated with ToLCNDV ORF AV2 mutants (Padidam *et al.*, 1996). Despite introducing point mutations at different positions within three ORF AV2 mutants, none of which affected the CP coding sequence, it was surprising to find that all mutants were unable to express detectable levels of CP. The fact that ssDNA accumulation was not significantly reduced for mutV2A indicates a phenotype distinct from that of the CP mutant, which is not attributable simply to a reduction in CP expression. It has been shown that mutants of the positional homologue (ORF V2) in BCTV produce an asymptomatic infection associated with elevated levels of dsDNA (Stanley *et al.*, 1992; Hormuzdi & Bisaro, 1993). In

this way, disruption of EACMZV ORF AV2 could have an indirect effect on CP accumulation by limiting the amount of ssDNA available for encapsidation, although the accumulation of ssDNA in plants infected with mutV2A suggests that this is not the case. The possibility that AV2 protein either plays a direct role in the control of CP expression or prevents CP turnover cannot be ruled out. Nonetheless, it is likely that expression of these two overlapping ORFs will be closely coordinated and it is conceivable that even subtle alterations could have significant effects on the spatial and/or temporal accumulation of CP, AV2 and viral DNA levels. It is worth noting that, although ACMV DNA-A virion-sense transcripts have been mapped (Townsend *et al.*, 1985), it is far from clear how CP expression occurs, particularly as a low-abundance transcript maps across the CP ORF while the major transcript maps across both virion-sense ORFs. Hence, it is also possible that the ORF AV2 mutations could impact on CP expression by affecting transcription or transcript processing.

ACMV AC4 has been implicated in counteracting the plant hypersensitive response to infection (van Wezel *et al.*, 2002), and has also been shown to suppress RNA silencing and induce developmental abnormalities in transgenic plants (Chellappan *et al.*, 2005). Furthermore, the AC4 protein homologue in monopartite begomoviruses and curtoviruses is an important symptom determinant and may be involved in virus movement (Rigden *et al.*, 1994; Latham *et al.*, 1997; Rojas *et al.*, 2001). Despite this, disruption of ACMV ORF AC4 had no effect on phenotype in *N. benthamiana* (Eteessami *et al.*, 1991). Here, we have demonstrated that an EACMZV ORF AC4 mutant is infectious, not only in *N. benthamiana* but also in cassava, without a significant change in phenotype. This suggests that considerable variation in AC4 function may exist between distinct begomoviruses or that there is an element of functional redundancy between EACMZV AC4 and other viral proteins (Vanitharani *et al.*, 2004), reflecting the considerable diversity observed within AC4 sequences of distinct species (Bull *et al.*, 2006).

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## REFERENCES

- Argüello-Astorga, G. R., Guevara-Gonzales, R. G., Herrera-Estrella, L. R. & Rivera-Bustamante, R. F. (1994). Geminivirus replication origins have a group-specific organization of iterative elements: a model for replication. *Virology* **203**, 90–100.
- Azzam, O., Frazer, J., de la Rosa, D., Beaver, J. S., Ahlquist, P. & Maxwell, D. P. (1994). Whitefly transmission and efficient ssDNA accumulation of bean golden mosaic geminivirus requires functional coat protein. *Virology* **204**, 289–296.

- Berrie, L. C., Rybicki, E. P. & Rey, M. E. C. (2001). Complete nucleotide sequence and host range of South African cassava mosaic virus: further evidence for recombination amongst begomoviruses. *J Gen Virol* **82**, 53–58.
- Bridson, R. W., Pinner, M. S., Stanley, J. & Markham, P. G. (1990). Geminivirus coat protein gene replacement alters insect specificity. *Virology* **177**, 85–94.
- Bridson, R. W., Liu, S., Pinner, M. S. & Markham, P. G. (1998). Infectivity of African cassava mosaic virus clones to cassava by biolistic inoculation. *Arch Virol* **143**, 2487–2492.
- Bull, S. E., Bridson, R. W., Sserubombwe, W. S., Ngugi, K., Markham, P. G. & Stanley, J. (2006). Genetic diversity and phylogeography of cassava mosaic viruses in Kenya. *J Gen Virol* **87**, 3053–3065.
- Chatterji, A., Chatterji, U., Beachy, R. N. & Fauquet, C. M. (2000). Sequence parameters that determine specificity of binding of the replication-associated protein to its cognate site in two strains of tomato leaf curl virus–New Delhi. *Virology* **273**, 341–350.
- Chellappan, P., Vanitharani, R. & Fauquet, C. M. (2004). Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J Virol* **78**, 7465–7477.
- Chellappan, P., Vanitharani, R. & Fauquet, C. M. (2005). MicroRNA-binding viral protein interferes with *Arabidopsis* development. *Proc Natl Acad Sci U S A* **102**, 10381–10386.
- Etessami, P., Watts, J. & Stanley, J. (1989). Size reversion of African cassava mosaic virus coat protein gene deletion mutants during infection of *Nicotiana benthamiana*. *J Gen Virol* **70**, 277–289.
- Etessami, P., Saunders, K., Watts, J. & Stanley, J. (1991). Mutational analysis of complementary-sense genes of African cassava mosaic virus DNA A. *J Gen Virol* **72**, 1005–1012.
- Fondong, V. N., Pita, J. S., Rey, M. E. C., de Kochko, A., Beachy, R. N. & Fauquet, C. M. (2000). Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon. *J Gen Virol* **81**, 287–297.
- Fontes, E. P. B., Luckow, V. A. & Hanley-Bowdoin, L. (1992). A geminivirus replication protein is a sequence-specific DNA binding protein. *Plant Cell* **4**, 597–608.
- Fontes, E. P. B., Eagle, P. A., Sipe, P., Luckow, V. A. & Hanley-Bowdoin, L. (1994a). Interaction between a geminivirus replication protein and origin DNA is essential for viral replication. *J Biol Chem* **269**, 8459–8465.
- Fontes, E. P. B., Gladfelter, H. J., Schaffer, R. L., Petty, I. T. D. & Hanley-Bowdoin, L. (1994b). Geminivirus replication origins have a modular organization. *Plant Cell* **6**, 405–416.
- Frischmuth, T., Roberts, S., von Arnim, A. & Stanley, J. (1993). Specificity of bipartite geminivirus movement proteins. *Virology* **196**, 666–673.
- Gibson, R. W., Legg, J. P. & Otim-Nape, G. W. (1996). Unusually severe symptoms are a characteristic of the current epidemic of mosaic virus disease of cassava in Uganda. *Ann Appl Biol* **128**, 479–490.
- Hanley-Bowdoin, L., Settlege, S. B., Orozco, B. M., Nagar, S. & Robertson, D. (1999). Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit Rev Plant Sci* **18**, 71–106.
- Harrison, B. D., Zhou, X., Otim-Nape, G. W., Liu, Y. & Robinson, D. J. (1997). Role of a novel type of double infection in the geminivirus-induced epidemic of severe cassava mosaic in Uganda. *Ann Appl Biol* **131**, 437–448.
- Hormuzdi, S. G. & Bisaro, D. M. (1993). Genetic analysis of beet curly top virus: evidence for three virion sense genes involved in movement and regulation of single- and double-stranded DNA levels. *Virology* **193**, 900–909.
- Klinkenberg, F. A. & Stanley, J. (1990). Encapsidation and spread of African cassava mosaic virus DNA A in the absence of DNA B when agroinoculated to *Nicotiana benthamiana*. *J Gen Virol* **71**, 1409–1412.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Latham, J. R., Saunders, K., Pinner, M. S. & Stanley, J. (1997). Induction of plant cell division by beet curly top virus gene C4. *Plant J* **11**, 1273–1283.
- Liu, S., Bridson, R. W., Bedford, I. D., Pinner, M. S. & Markham, P. G. (1998). Identification of genes directly and indirectly involved in the insect transmission of African cassava mosaic geminivirus by *Bemisia tabaci*. *Virus Genes* **18**, 5–11.
- Maruthi, M. N., Colvin, J., Seal, S. & Thresh, J. M. (2002). First report of a distinct begomovirus infecting cassava from Zanzibar. *Plant Dis* **86**, 187.
- Maruthi, M. N., Seal, S., Colvin, J., Bridson, R. W. & Bull, S. E. (2004). East African cassava mosaic Zanzibar virus – a recombinant begomovirus species with a mild phenotype. *Arch Virol* **149**, 2365–2377.
- Morris, B., Richardson, K., Eddy, P., Zhan, X., Haley, A. & Gardner, R. (1991). Mutagenesis of the AC3 open reading frame of African cassava mosaic virus DNA A reduces DNA B replication and ameliorates disease symptoms. *J Gen Virol* **72**, 1205–1213.
- Ogbe, F. O., Thottappilly, G., Dixon, A. G. O., Atiri, G. I. & Mignouna, H. D. (2003). Variants of East African cassava mosaic virus and its distribution in double infections with African cassava mosaic virus in Nigeria. *Plant Dis* **87**, 229–232.
- Orozco, B. M., Gladfelter, H. J., Settlege, S. B., Eagle, P. A., Gentry, R. N. & Hanley-Bowdoin, L. (1998). Multiple *cis* elements contribute to geminivirus origin function. *Virology* **242**, 346–356.
- Padidam, M., Beachy, R. N. & Fauquet, C. M. (1995). Tomato leaf curl geminivirus from India has a bipartite genome and coat protein is not essential for infectivity. *J Gen Virol* **76**, 25–35.
- Padidam, M., Beachy, R. N. & Fauquet, C. M. (1996). The role of AV2 ('precoat') and coat protein in viral replication and movement in tomato leaf curl geminivirus. *Virology* **224**, 390–404.
- Padidam, M., Sawyer, S. & Fauquet, C. M. (1999). Possible emergence of new geminiviruses by frequent recombination. *Virology* **265**, 218–225.
- Pita, J. S., Fondong, V. N., Sangaré, A., Otim-Nape, G. W., Ogwal, S. & Fauquet, C. M. (2001). Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. *J Gen Virol* **82**, 655–665.
- Pooma, W., Gillette, W. K., Jeffrey, J. L. & Petty, I. T. D. (1996). Host and viral factors determine the dispensability of coat protein for bipartite geminivirus systemic movement. *Virology* **218**, 264–268.
- Rigden, J. E., Krake, L. R., Rezaian, M. A. & Dry, I. B. (1994). ORF C4 of tomato leaf curl geminivirus is a determinant of symptom severity. *Virology* **204**, 847–850.
- Roberts, S. & Stanley, J. (1994). Lethal mutations within the conserved stem-loop of African cassava mosaic virus DNA are rapidly corrected by genomic recombination. *J Gen Virol* **75**, 3203–3209.
- Rojas, M. R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M. R., Lucas, W. J. & Gilbertson, R. L. (2001). Functional analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf curl virus. *Virology* **291**, 110–125.
- Rothstein, D., Bridson, R. W., Haible, D., Stanley, J., Frischmuth, T. & Jeske, H. (2005). Biolistic infection of cassava using cloned

- components of Indian cassava mosaic virus. *Arch Virol* **150**, 1669–1675.
- Saunders, K., Bedford, I. D. & Stanley, J. (2001).** Pathogenicity of a natural recombinant associated with ageratum yellow vein disease: implications for begomovirus evolution and disease aetiology. *Virology* **282**, 38–47.
- Saunders, K., Salim, N., Mali, V. R., Malathi, V. G., Briddon, R., Markham, P. G. & Stanley, J. (2002).** Characterisation of Sri Lankan cassava mosaic virus and Indian cassava mosaic virus: evidence for acquisition of a DNA B component by a monopartite begomovirus. *Virology* **293**, 63–74.
- Stanley, J. (1983).** Infectivity of the cloned geminivirus genome requires sequences from both DNAs. *Nature* **305**, 643–645.
- Stanley, J. & Gay, M. R. (1983).** Nucleotide sequence of cassava latent virus DNA. *Nature* **301**, 260–262.
- Stanley, J. & Townsend, R. (1986).** Infectious mutants of cassava latent virus generated *in vivo* from intact recombinant clones containing single copies of the genome. *Nucleic Acids Res* **14**, 5981–5998.
- Stanley, J., Frischmuth, T. & Ellwood, S. (1990).** Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. *Proc Natl Acad Sci U S A* **87**, 6291–6295.
- Stanley, J., Latham, J. R., Pinner, M. S., Bedford, I. & Markham, P. G. (1992).** Mutational analysis of the monopartite geminivirus beet curly top virus. *Virology* **191**, 396–405.
- Stanley, J., Bisaro, D. M., Briddon, R. W., Brown, J. K., Fauquet, C. M., Harrison, B. D., Rybicki, E. P. & Stenger, D. C. (2005).** *Geminiviridae*. In *Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 301–326. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London: Elsevier/Academic Press.
- Thresh, J. M. & Cooter, R. J. (2005).** Strategies for controlling cassava mosaic virus disease in Africa. *Plant Pathol* **54**, 587–614.
- Thresh, J. M., Fishpool, L. D. C., Otim-Nape, G. W. & Fargette, D. (1994).** African cassava mosaic virus disease: an underestimated and unsolved problem. *Tropical Sci* **34**, 3–14.
- Townsend, R., Stanley, J., Curson, S. J. & Short, M. N. (1985).** Major polyadenylated transcripts of cassava latent virus and location of the gene encoding coat protein. *EMBO J* **4**, 33–37.
- Unsel, S., Höhnle, M., Ringel, M. & Frischmuth, T. (2001).** Subcellular targeting of the coat protein of African cassava mosaic geminivirus. *Virology* **286**, 373–383.
- Unsel, S., Frischmuth, T. & Jeske, H. (2004).** Short deletions in nuclear targeting sequences of African cassava mosaic virus coat protein prevent geminivirus twinned particle formation. *Virology* **318**, 90–101.
- Vanitharani, R., Chellappan, P., Pita, J. S. & Fauquet, C. M. (2004).** Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J Virol* **78**, 9487–9498.
- van Wezel, R., Dong, X., Blake, P., Stanley, J. & Hong, Y. (2002).** Differential roles of geminivirus Rep and AC4 (C4) in the induction of necrosis in *Nicotiana benthamiana*. *Mol Plant Pathol* **3**, 461–471.
- von Arnim, A. & Stanley, J. (1992).** Determinants of tomato golden mosaic virus symptom development located on DNA B. *Virology* **186**, 286–293.
- von Arnim, A., Frischmuth, T. & Stanley, J. (1993).** Detection and possible functions of African cassava mosaic virus DNA B gene products. *Virology* **192**, 264–272.
- Ward, A., Etessami, P. & Stanley, J. (1988).** Expression of a bacterial gene in plants mediated by infectious geminivirus DNA. *EMBO J* **7**, 1583–1587.
- Wartig, L., Khey-Pour, A., Noris, E., de Kouchovsky, F., Jouanneau, F., Gronenborn, B. & Jupin, I. (1997).** Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. *Virology* **228**, 132–140.
- Zhang, P. & Grisse, W. (2003).** Efficient replication of cloned African cassava mosaic virus in cassava leaf disks. *Virus Res* **92**, 47–54.
- Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G. W., Robinson, D. J. & Harrison, B. D. (1997).** Evidence that DNA A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *J Gen Virol* **78**, 2101–2111.