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Characterisation of Sri Lankan Cassava Mosaic Virus and Indian Cassava Mosaic Virus: Evidence for Acquisition of a DNA B Component by a Monopartite Begomovirus

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Two bipartite begomoviruses, Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV), have been isolated from mosaic-diseased cassava originating from central India and Sri Lanka, respectively. ICMV was transmitted with low efficiency from cassava to *Nicotiana benthamiana* by sap inoculation to give leaf curl symptoms. SLCMV was much more virulent in this host, producing severe stunting, leaf curl, and chlorosis. These symptoms were reproduced when their cloned genomic components (DNAs A and B) were introduced into *N. benthamiana* by either mechanical or *Agrobacterium*-mediated inoculation (agroinoculation). SLCMV is more closely related to ICMV (DNA A, 84%; DNA B, 94% nucleotide identity) than African cassava mosaic virus (ACMV) (DNA A, 74%; DNA B, 47% nucleotide identity). Sequence comparisons suggest that SLCMV DNA B originated from ICMV DNA B by a recombination event involving the SLCMV DNA A intergenic region. Pseudorecombinants produced by reassortment of the cloned components of ICMV and ACMV were not infectious in *N. benthamiana,* emphasising their status as distinct virus species. In contrast, a pseudorecombinant between ACMV DNA A and SLCMV DNA B was infectious. Consistent with these observations, iteron motifs located within the intergenic region that may be involved in the initiation of viral DNA replication are conserved between SLCMV and ACMV but not ICMV. When introduced into *N. benthamiana* by agroinoculation, SLCMV DNA A alone produced a severe upward leaf roll symptom, reminiscent of the phenotype associated with some monopartite begomoviruses. Furthermore, coinoculation of SLCMV DNA A and the satellite DNA β associated with ageratum yellow vein virus (AYVV) produced severe downward leaf curl in *N. glutinosa* and yellow vein symptoms in *Ageratum conyzoides,* resembling the phenotypes associated with AYVV DNA A and DNA β infection in these hosts. Thus, SLCMV DNA A has biological characteristics of a monopartite begomovirus, and the virus probably evolved by acquisition of a DNA B component from ICMV.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), belonging to the family Euphorbiaceae, is the third most important food crop after cereals and legumes grown in the tropics. Cassava mosaic disease (CMD) occurs at high incidence wherever cassava is grown in Africa and India and is considered as one of the major constraints in cultivation of this crop (Malathi *et al.,* 1985; Fauquet and Fargette, 1990). *African cassava mosaic virus* (ACMV; family *Geminiviridae,* genus *Begomovirus*) (Bock *et al.,* 1978; Stanley and Gay, 1983) was the original virus species to be associated with CMD in Africa, although two other distinct begomovirus species, *East African cassava mosaic virus* (EACMV) and *South African cassava mosaic virus* (SACMV), are now recognised (Zhou *et al.,* 1998; Berrie *et al.,* 2001). A fourth species, *Indian cassava mosaic virus* (ICMV), is associated with CMD in India (Hong *et al.,* 1993).

All begomoviruses that infect cassava are typical of the majority of members of the genus, having genomes comprising two circular single-stranded DNA components (DNAs A and B) (Stanley, 1983; Stanley and Gay, 1983). Reintroduction of cloned DNA A and DNA B components of ACMV and SACMV into cassava by either biolistic delivery or *Agrobacterium tumefaciens*-mediated inoculation (agroinoculation) to produce CMD symptoms confirmed the aetiology of the disease (Briddon *et al.,* 1998; Berrie *et al.,* 2001). In contrast, the begomoviruses tomato yellow leaf curl virus (TYLCV; Kheyr-Pour *et al.,* 1991; Navot *et al.,* 1991), tomato leaf curl virus (ToLCV; Dry *et al.,* 1993), ageratum yellow vein virus (AYVV; Tan *et al.,* 1995), and cotton leaf curl virus (CLCuV; Briddon *et al.,* 2000) have only a single genomic component resembling DNA A. Of these, AYVV and CLCuV have associated satellite DNA components (referred to as DNA β) that are essential for maintenance of the disease (Saunders *et al.,* 2000; Briddon *et al.,* 2001).

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Saunders *et al.* (2000) suggested that a small satellite DNA associated with ToLCV infection (Dry *et al.,* 1997) may also be a remnant of a DNA β component. So far, there have been no reports of such satellite components being associated with bipartite begomoviruses.

Geminiviruses are highly recombinogenic, as demonstrated by their propensity for rearrangement of their genomic DNA (Etessami *et al.,* 1989) and exchange of sequences between genomic components (Roberts and Stanley, 1994). Interspecific recombination events have probably contributed significantly to begomovirus diversity (Padidam *et al.,* 1999), and recombination with members of other genera (Stanley *et al.,* 1986; Briddon *et al.,* 1996) and families (Saunders and Stanley, 1999; Saunders *et al.,* 2001) has been reported. Begomoviruses that infect cassava are known to undergo interspecific recombination, and such events between ACMV and EACMV are believed to be responsible for a severe outbreak of CMD in Uganda (Zhou *et al.,* 1997; Fondong *et al.,* 2000; Pita *et al.,* 2001).

Here, we have isolated and characterised two distinct bipartite begomoviruses associated with CMD-affected cassava on the Indian subcontinent. One virus, associated with cassava growing in central India, is an isolate of ICMV. The other, from Sri Lanka, is considered to be an isolate of a distinct species that we have tentatively named Sri Lankan cassava mosaic virus (SLCMV). We present evidence to show that the SLCMV DNA A component has characteristics of a monopartite begomovirus and suggest that it has evolved into a bipartite virus by recombination with ICMV.

RESULTS

Mechanical transmission of ICMV and SLCMV isolates

ICMV was transmitted with low efficiency from cassava to *Nicotiana benthamiana* (1/20 inoculated plants) by mechanical inoculation. The infected plant developed systemic symptoms of downward curling of the upper leaves 2–3 weeks after inoculation, resulting in slight stunting of the plant. These symptoms resemble those reported for an isolate of ICMV from southern India (Mathew and Muniyappa, 1992). The virus was also transmitted with low efficiency between *N. benthamiana* plants (3/100 inoculated plants over two experiments) to produce similar mild symptoms. SLCMV was transmitted from cassava to *N. benthamiana* at a higher efficiency (3/6 and 5/6 inoculated plants using frozen and fresh tissues, respectively, as the virus source), and plants developed symptoms between 3 and 5 weeks after inoculation. In contrast to ICMV, SLCMV was mechanically transmitted with high efficiency between *N. benthamiana* plants (28/28 inoculated plants) and produced severe downward leaf curl, chlorosis, and stunting 1 week after inoculation. Unlike ICMV, the symptoms associated with SLCMV infection are similar to ACMV infection of this host (Stanley, 1983).

Infectivity of ICMV and SLCMV cloned DNA

Full-length copies of ICMV and SLCMV DNA A and DNA B components were cloned from viral supercoiled DNA (scDNA) isolated from infected plants. In a preliminary experiment, the inserts of ICMV clones pICM15 and pICM18 were shown to be infectious when mechanically coinoculated onto *N. benthamiana* plants, although with low efficiency (1/5 inoculated plants), as observed for the wild-type isolate. Furthermore, symptoms were identical to those associated with the wild-type isolate in this host. In view of the low infectivity of both virus and cloned DNA following mechanical inoculation, the host range of the virus was further investigated by agroinoculation. *N. benthamiana* was highly susceptible to ICMV when pBinICM1.6A, containing a partial repeat of DNA A, was coinoculated with either pBinICM1.6B or pBinICM1.4B, containing partial repeats of DNA B (12/22 and 20/28 inoculated plants, respectively), and these clones induced typical symptoms (Fig. 1B). However, the efficiency of sap transmission of progeny virus from agroinoculated plants to healthy *N. benthamiana* remained low (4/140 inoculated plants). The clone combinations induced severe leaf curl and mild chlorosis in *N. clevelandii* (9/13 and 18/22 inoculated plants, respectively) and leaf curl and a network of veinal chlorosis in *N. glutinosa* (8/13 and 14/22 inoculated plants, respectively). We were unable to infect *N. rustica, N. tabacum* (cv Xanthi), and *Datura stramonium* with ICMV, although all of these plant species are susceptible to ACMV. Although ACMV DNA A alone can systemically infect *N. benthamiana* when introduced by agroinoculation (Klinkenberg and Stanley, 1990), neither ACMV nor ICMV DNA A can induce symptoms in this host in the absence of its cognate DNA B component.

The inserts of SLCMV clones pSL1 and pSL4 were highly infectious when introduced into *N. benthamiana* by mechanical inoculation (5/5 inoculated plants) and produced chlorotic lesions on the inoculated leaves after 5 days and systemic symptoms typical of the wild-type isolate after 10 days. When introduced into this host by agroinoculation, SLCMV clones pBinSLCM1.6A and pBinSLCM1.8B, containing partial repeats of DNA A and DNA B, respectively, produced typical systemic symptoms after 7 days (Fig. 1A). In addition, SLCMV DNA A induced upward leaf roll and vein swelling symptoms when agroinoculated on its own into *N. benthamiana* (5/5 inoculated plants; Fig. 1C). These symptoms are indistinguishable from those induced in this host by the monopartite begomovirus AYVV (Tan *et al.,* 1995; Stanley *et al.,* 1997).

SLCMV was successfully reintroduced into cassava by biolistic inoculation of the pSL1 and pSL4 inserts (3/3

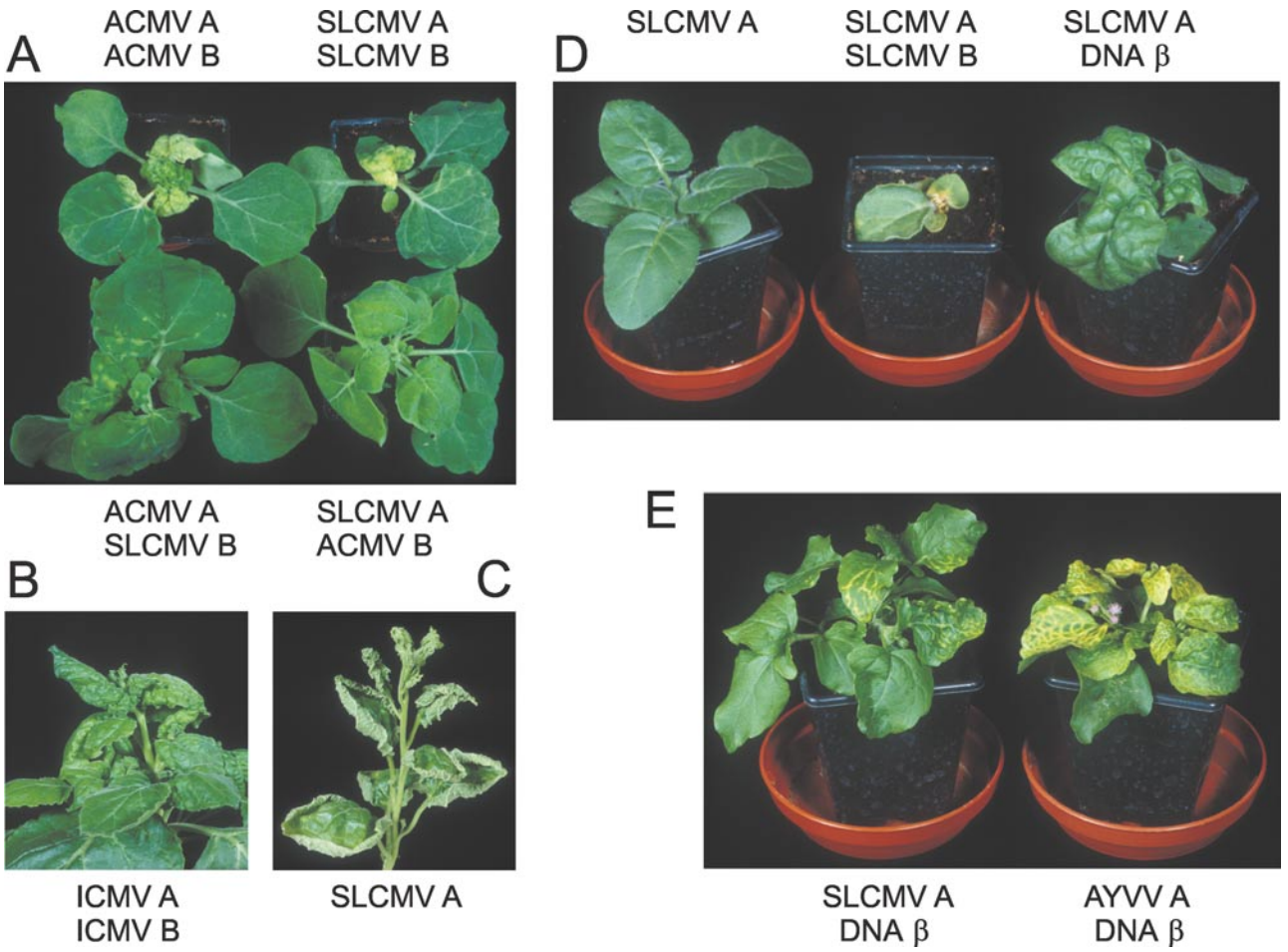

FIG. 1. Symptoms associated with SLCMV, ICMV, ACMV, and their pseudorecombinants. (A) ACMV (top left), SLCMV (top right), and their pseudorecombinants (bottom left and right) in *N. benthamiana* at 14 dpi. (B) ICMV in *N. benthamiana* at 25 dpi. (C) SLCMV DNA A alone in *N. benthamiana* at 38 dpi. (D) SLCMV DNA A alone (left) and in the presence of either DNA B (centre) or AYVV satellite DNA β (right) in *N. glutinosa* at 17 dpi. (E) SLCMV DNA A and DNA β (left) and AYVV DNA A and DNA β (right) in *A. conyzoides* at 17 dpi. In all cases, virus components were introduced into plants by agroinoculation.

inoculated plants) to produce leaf curl and mosaic symptoms approximately 6 weeks after inoculation. The presence of viral single- and double-stranded DNA forms in these plants was verified by Southern blot analysis (data not shown). Our initial attempts to reintroduce the cloned components of ICMV into cassava, either biolistically or by agroinoculation, have proved unsuccessful, although it has been achieved for ACMV and SACMV by these methods (Briddon *et al.,* 1998; Berrie *et al.,* 2001). The reason for this is unknown but may be due either to the less aggressive nature of ICMV compared with ACMV and SLCMV, as demonstrated by mechanical inoculation to *N. benthamiana,* or to a defect in the clones as previously reported for an isolate of ACMV (Briddon *et al.,* 1998). It has been argued that repeated passaging through *N. benthamiana* before cloning the genomic components may select a virus population that can no longer infect cassava (Liu *et al.,* 1997). However, this is unlikely to be the case for the isolate of ICMV described here as it was maintained only briefly in *N. benthamiana* prior to extraction of the viral scDNA. The lack of ICMV infectivity in cassava is the subject of a current investigation.

Derivation and comparison of ICMV and SLCMV nucleotide sequences

The complete nucleotide sequences of infectious cloned DNA A and DNA B components of ICMV (pICM15 and pICM18) and SLCMV (pSL1 and pSL4) have been determined. The sequences appear in the EMBL Nucleotide Sequence Database under Accession Nos. AJ314737–AJ314740. Both viruses encode two virion-sense (AV1 and AV2) and four complementary-sense (AC1, AC2, AC3, and AC4) open reading frames (ORFs) on DNA A and one virion-sense (BV1) and one complementary-sense (BC1) ORF on DNA B that are normally found in bipartite begomoviruses from the Old World. The di-

vergent ORFs on complementary strands are separated by an intergenic region containing a common region that is conserved to some extent (discussed below) between components. The common region contains a stem-loop sequence and TAATATTAC nonanucleotide found in all geminiviruses. Sequences are numbered starting from the nucleotide located immediately downstream of the virion-sense strand nick site (↓) within the TAATATT↓AC motif according to current convention.

The ICMV nucleotide sequence is most closely related (DNA A, 98.4% identity; DNA B, 91.9% identity; excluding the repeat sequences described below) to that of the previously characterised ICMV isolate from southern India (Hong *et al.,* 1993). It is clearly an isolate of ICMV, and we have named it ICMV-[Mah] to indicate that it originated from Maharashtra state and to distinguish it from the original isolate, ICMV-[Ker], from Kerala state. The major difference between the components of these two isolates resides in the sequences immediately downstream of the stem-loop region that, for ICMV-[Ker], contains an extensive 41-bp triple repeat within its common region. The ICMV-[Mah] components have only a single copy of this sequence. The significance of the ICMV-[Ker] repeat is unknown as the biological activity of the cloned components has not been investigated for this isolate. The repeat serves to extend the common region from nucleotide 73 in ICMV-[Mah] to nucleotide 267 in ICMV-[Ker]. Similar to the ICMV-[Ker] common region, that of ICMV-[Mah] is highly conserved (99.6% identity) between its components, having only one mismatch between nucleotides 2605-73 (DNA A) and 2585-73 (DNA B). The presence of the repeats is largely responsible for the size difference between the DNA A components (ICMV-[Mah], 2739 nucleotides; ICMV-[Ker], 2815 nucleotides). Comparison of the DNA B components indicates that two copies of the ICMV-[Ker] repeat have been replaced with a larger and unrelated sequence extending from ICMV-[Mah] nucleotides 74 to 318. Together with other deletions and insertions (notably a 21-bp insert in ICMV-[Mah] upstream of ORF BC1 between nucleotides 2444 and 2464), this unrelated sequence contributes to the size difference between the DNA B components (ICMV-[Mah], 2719 nucleotides; ICMV-[Ker], 2645 nucleotides). All other sequences are highly conserved (96.7% identity), suggesting that the unrelated sequence has been inserted into the ICMV-[Mah] component as a result of a recombination event. The origin of this fragment is unknown, and a database search has failed to show any extensive homology to other geminivirus sequences.

Comparison with other geminiviruses shows that SLCMV is most closely related to ICMV-[Mah], having 84.2 and 94.2% nucleotide sequence identity between their DNA A and DNA B components, respectively. Similar to ICMV-[Mah], the SLCMV components do not have extensive repeat sequences within the common region. The SLCMV common region is less well conserved between its components (89.2% identity) than that of ICMV, although sequences upstream of the stem-loop are better conserved (96.3% identity) than those downstream (66.7% identity) (Fig. 2B). However, it is noticeable that sequences upstream of the stem-loop, including the 5′-terminus of the gene encoding the replication-associated protein (Rep), are less well conserved between SLCMV and ICMV-[Mah] DNA A components. Indeed, a database search revealed that the SLCMV common region sequences upstream of the stem-loop are most closely related to those of ACMV (77.8% identity) (Fig. 2A). A more detailed inspection of this region reveals that both SLCMV components contain the duplicated iteron motif AATTGGAGACA that is identical to that found in ACMV (Fig. 2A). A sequence resembling this motif (AAcTaGAGACA) is additionally present in a short insert located between the two ACMV iterons. In contrast, ICMV-[Mah] has the iteron GGTACTCA located in the same relative position within its common region.

Comparison of SLCMV and ICMV-[Mah] DNA B components indicates that sequences outside of their common regions are very highly conserved (97.2% identity). Begomovirus DNA B components are generally less well conserved than DNA A components, and this level of DNA B sequence identity is comparable to that associated with some closely related isolates. For example, Kenyan and Nigerian isolates of ACMV (ACMV-[KE], Accession No. J02058, and ACMV-[NG], Accession No. X17096) show 94.0% identity. However, the SLCMV and ICMV-[Mah] DNA B common region sequences are not so conserved, with sequences upstream of the stem-loop being less so (59.2% identity) than those downstream (80.7% identity) (Fig. 2B). In view of the relatively low level of nucleotide identity between SLCMV common region sequences downstream of the stem-loop, these observations are consistent with a recombination event in which part of the ICMV DNA B common region, upstream of the stem-loop, has been replaced with the equivalent region from SLCMV DNA A. This event may have been mediated by the nick site within the origin of replication that is considered to be a recombination hot spot (Etessami *et al.,* 1989; Stanley, 1995; Saunders and Stanley, 1999). As a result of this recombination event, SLCMV DNA A will have transferred virus-specific *cis*-acting motifs required for replication, enabling it to capture the ICMV DNA B component.

To investigate whether the SLCMV sequences were representative, sequences encompassing the common region of additional DNA A and DNA B clones pSL6 and pSL9, respectively, were derived. DNA A nucleotides 2616–134 were identical to those of clone pSL1 with the exception of an extra cytidine residue within the run of six cytidine residues (nucleotides 34–39). DNA B nucleotides 2057–198 were identical to clone pSL4.

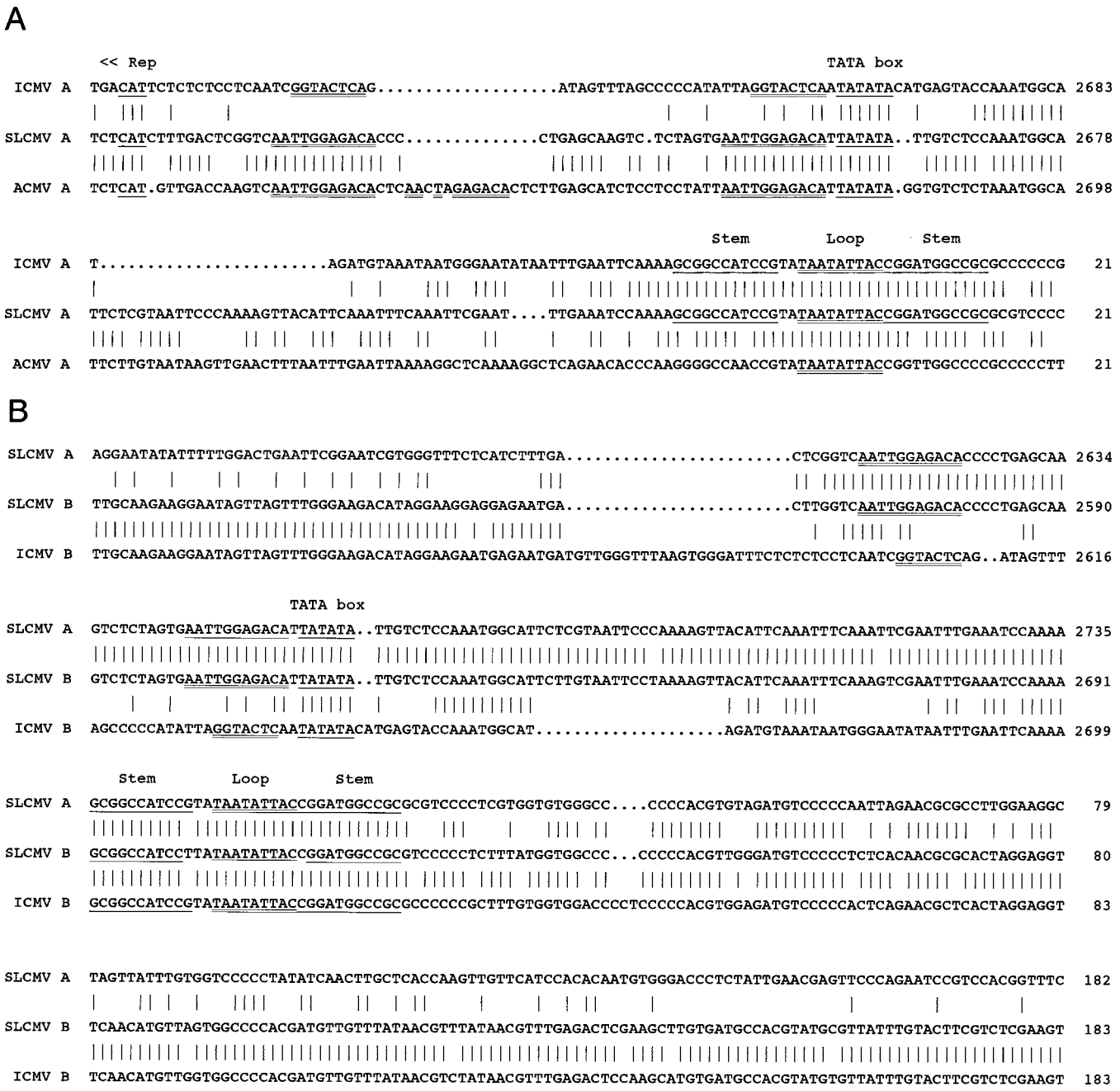

FIG. 2. Sequence comparison of intergenic regions of cassava-infecting begomoviruses. (A) Comparison of DNA A components of ICMV, SLCMV, and ACMV showing the location of iterons (double underlined) between the putative TATA box and start of the Rep open reading frame, all located upstream of the stem-loop structure. (B) Comparison of ICMV DNA B with the common region and flanking sequences of SLCMV DNA A and DNA B.

Infectivity of ICMV, SLCMV, and ACMV pseudorecombinants

In view of their nucleotide sequence relationships, we were interested to know if ICMV, SLCMV, and ACMV could form viable pseudorecombinants by the reassortment of their cloned genomic components. Infectivity was assayed by agroinoculation of their common host *N. benthamiana* (Table 1), and transreplication of components was determined using a *N. benthamiana* leaf disk assay. Pseudorecombinants produced by the exchange of ICMV and ACMV components were not infectious as judged by the lack of symptom development and inability to detect viral DNA in newly emerging leaves by dot-blot analysis. ICMV, SLCMV, and ACMV induced typical symptoms in control experiments performed at the same time. A single plant agroinoculated with the combination ICMV DNA A and SLCMV DNA B became systemically infected and developed leaf curl and chlorotic symptoms resembling a mild SLCMV infection. Dot-blot analysis demonstrated that both components were present in this

TABLE 1

Infectivity of Pseudorecombinants in *N. benthamiana*

Inoculum	Plants			Symptoms
	Expt. 1	Expt. 2	Expt. 3	
SLCMV A + SLCMV B	—	6/6	1/1	Severe stunting, leaf curl, chlorosis
SLCMV A + ICMV B	—	6/6	4/4	Leaf roll, vein swelling
SLCMV A + ACMV B	—	6/6	4/4	Leaf roll, vein swelling
ICMV A + ICMV B	14/20	2/6	3/4	Stunting, leaf curl
ICMV A + SLCMV B	—	0/6	1/4	Mild stunting, leaf curl
ICMV A + ACMV B	0/20	0/6	0/4	—
ACMV A + ACMV B	20/20	6/6	1/1	Severe stunting, leaf curl, chlorosis
ACMV A + SLCMV B	—	6/6	4/4	Stunting, leaf curl, veinal chlorosis
ACMV A + ICMV B	0/20	0/6	0/4	—

plant. In contrast, the combination ACMV DNA A and SLCMV DNA B produced systemic symptoms of stunting, leaf curl, and chlorosis in all agroinoculated plants. Symptoms were generally milder than those induced by either ACMV or SLCMV (Fig. 1A), with chlorotic spots occurring on the first symptomatic leaves, developing into veinal chlorosis later in infection. Frequently, plants eventually developed a general chlorosis resembling the parental virus symptoms. Southern blot analysis confirmed that plants infected with ACMV DNA A and SLCMV DNA B contained both genomic components (data not shown). Plants agroinoculated with SLCMV DNA A and either ACMV DNA B or ICMV DNA B invariably produced upward leaf roll and vein swelling symptoms typical of SLCMV DNA A alone (a mild phenotype, typical of early infection, is shown in Fig. 1A). Southern blot analysis showed that plants inoculated with these combinations contained only SLCMV DNA A (data not shown).

Because begomoviruses are highly recombinogenic, we wished to investigate if recombination events were responsible for pseudorecombinant viability. Accordingly, nucleic acids were extracted from two randomly chosen plants systemically infected with ACMV DNA A and SLCMV DNA B, and from the single plant infected with ICMV DNA A and SLCMV DNA B. An ~1600-bp fragment of SLCMV DNA B encompassing the entire common region was PCR-amplified from each extract and purified. Digestion of the fragments derived from plants infected with ACMV DNA A and SLCMV DNA B with *Eco*RI produced fragments of ~1100 and ~500 bp expected for wild-type SLCMV DNA B (*Eco*RI sites occur at positions 2449 and 2465). Sequence analysis of four clones, two originating from each extract, confirmed that SLCMV DNA B had retained its original sequence, indicating that recombination with ACMV DNA A had not occurred. In contrast, the fragment derived from the single plant infected with ICMV DNA A and SLCMV DNA B produced two fragments of ~900 and ~700 bp, consistent with the replacement of the two SLCMV DNA B *Eco*RI sites with a single site situated immediately upstream of the stem-loop in the common region, as occurs in ICMV DNA A at position 2710. Analysis of four clones revealed that they had identical sequences that were consistent with a recombination event in which nucleotides 2299/2302–2703/14 of SLCMV DNA B had been replaced with nucleotides 2337/2340–2730/14 of ICMV DNA A. The exact recombination points cannot be determined due to the presence of identical sequences at these positions in both components. However, the downstream recombination point encompasses the proposed recombinational hot spot corresponding to the nick site within the origin of replication. The fragment of ICMV DNA A that has been inserted into SLCMV DNA B contains putative *cis*-acting motifs required for ICMV Rep binding.

N. benthamiana leaf disk assays (Fig. 3) demonstrated that ACMV DNA A transreplicated DNA B of SLCMV but not that of ICMV, SLCMV DNA A transreplicated ACMV DNA B but not that of ICMV, and ICMV DNA A was unable to transreplicate either ACMV or SLCMV DNA B. These results are consistent with the infectivity data of pseudorecombinants in plants. During one such experiment, SLCMV DNA A was observed to transreplicate ICMV DNA B. Because this was not reproducible, it is assumed that transreplication on this occasion resulted from a recombination event similar to that described above. Attempts to confirm this by PCR amplification of a DNA B fragment using primers that should selectively amplify a fragment from *de novo* replicated DNA B rather than the residual inoculum have been unsuccessful, possibly due to the low level of accumulation of the replicating DNA.

Interaction between SLCMV DNA A and a satellite DNA component

Having demonstrated that SLCMV DNA A alone can cause a symptomatic infection in *N. benthamiana* that is identical to AYVV DNA A infection of this host, we were

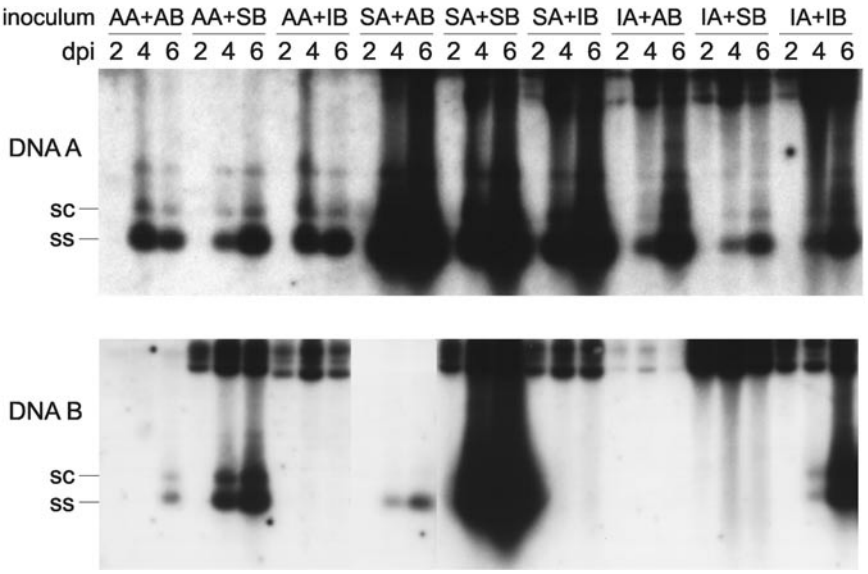

FIG. 3. Southern blot analysis of the replication of ACMV, SLCMV, ICMV, and their pseudorecombinants in *N. benthamiana* leaf disks. Leaf disks were agroinoculated with various combinations of virus components (inoculum; the first letter refers to ACMV (A), SLCMV (S), and ICMV (I), the second letter refers to the genomic component (A and B)), and nucleic acids were extracted 2, 4, and 6 dpi. Blots were hybridised to DNA A (top) and DNA B (bottom) probes lacking common region sequences to avoid cross-hybridisation. Because different fragments were used to probe for each component, the relative intensities of the signals cannot be directly compared. The positions of single-stranded (ss) and supercoiled (sc) DNAs are indicated.

interested to know if SLCMV DNA A could functionally interact with the satellite DNA β associated with ageratum yellow vein disease. To investigate this possibility, *N. glutinosa* plants were agroinoculated with various clone combinations. SLCMV induced severe stunting, leaf curl, and chlorosis in this host (5/5 plants inoculated; Fig. 1D). SLCMV DNA A alone caused mild stunting associated with extremely mild leaf curl symptoms (5/5 plants inoculated). However, coinoculation of SLCMV DNA A and AYVV DNA β produced severe stunting, leaf curl, and vein greening symptoms (5/5 plants inoculated) that were only slightly less severe that those caused by AYVV DNA A and DNA β in this host (Fig. 1D; Saunders *et al.,* 2001). DNA β accumulation in these plants was verified by Southern blot analysis of extracts of systemically infected tissue from two plants chosen at random (Fig. 4). In contrast, plants coinoculated with AYVV DNA β and DNA A of either ACMV or ICMV remained asymptomatic, and viral DNAs were not detected in the upper leaves by dot-blot analysis (16 plants screened for each combination). To investigate their ability to transreplicate DNA β, *N. benthamiana* leaf disks were coinoculated with DNA β and DNA A of either SLCMV, ACMV, or ICMV (Fig. 5). DNA β was transreplicated by both ACMV and ICMV DNA A in addition to SLCMV DNA A under these conditions.

Although SLCMV is unable to systemically infect *A. conyzoides,* as judged by the lack of systemic symptoms (eight plants inoculated) and inability to detect viral DNA by dot-blot analysis (six plants screened), we were interested to know if the ability of SLCMV DNA A to interact with AYVV DNA β could change its host range. Agroinoculation of SLCMV DNA A with AYVV DNA β caused a systemic infection of *A. conyzoides,* albeit with relatively low efficiency (2/8 inoculated plants), to produced a mild yellow vein phenotype (Fig. 1E).

The high level of coinfection of SLCMV DNA A and AYVV DNA β in *N. glutinosa* suggests that their functional interaction is not the result of a recombination event in which the SLCMV DNA A replication origin had been inserted into DNA β. To confirm this, an ~850-bp fragment of DNA β encompassing the putative intergenic

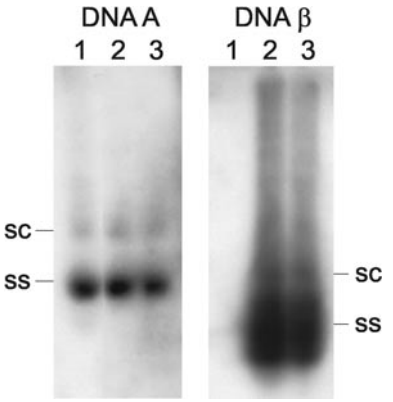

FIG. 4. Southern blot analysis of AYVV satellite DNA β systemic infection of *N. glutinosa* in the presence of SLCMV DNA A. Plants were agroinoculated with either SLCMV DNA A alone (lane 1) or SLCMV DNA A and AYVV satellite DNA β (lanes 2 and 3). Blots were hybridised to DNA A (left) and DNA β (right) probes. The positions of single-stranded (ss) and supercoiled (sc) DNAs are indicated.

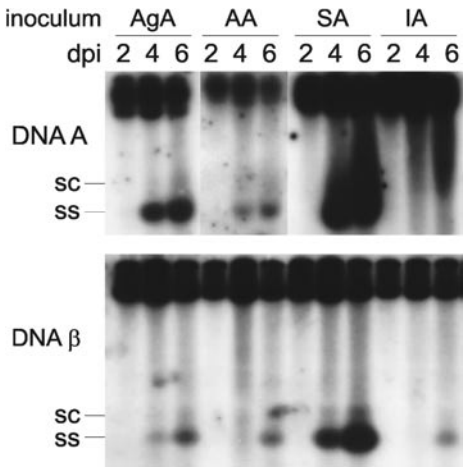

FIG. 5. Southern blot analysis of AYVV satellite DNA β trans-replication by DNA A of AYVV, ACMV, SLCMV, and ICMV in *N. benthamiana* leaf disks. Leaf disks were co-agroinoculated with DNA β and DNA A of either AYVV (AgA), ACMV (AA), SLCMV (SA), or ICMV (IA), and nucleic acids were extracted 2, 4, and 6 dpi. Blots were hybridised to the appropriate DNA A (top) and DNA β (bottom) probes. Because different fragments were used to probe for each component, the relative intensities of the signals cannot be directly compared. On this occasion, and in contrast to the data of Fig. 3, ICMV DNA A occurs mainly as high molecular weight DNA. The reason for this is unclear. The positions of single-stranded (ss) and supercoiled (sc) DNAs are indicated.

region was PCR-amplified and cloned from extracts of infected *N. glutinosa* and *A. conyzoides* plants. Analysis of a single clone originating from *N. glutinosa* and three clones from *A. conyzoides* indicated that wild-type DNA β sequences had been retained and, hence, that recombination had not occurred.

DISCUSSION

We have isolated and characterised two distinct bipartite begomoviruses originating from cassava growing in India and Sri Lanka. On the basis of host range, phenotype, genome organisation, and sequence homology, the virus from Maharashtra state in the west of India (ICMV-[Mah]) is considered to be a closely related isolate of a previously characterised virus from Kerala state in southern India (ICMV-[Ker]) (Hong *et al.,* 1993). The main difference between these isolates is the presence of large direct repeats in the common region of ICMV-[Ker]. Although absent in ICMV-[Mah], the repeats are not required for infectivity of this isolate and their function remains unknown. The virus from Sri Lanka, that we have named SLCMV, is most closely related to ICMV. However, we consider it to be a new species on the basis of criteria outlined by the ICTV (Rybicki *et al.,* 2000), specifically on the grounds of DNA A sequence diversity (84.2% identity with ICMV DNA A), difference in phenotype when compared with ICMV, inability to form viable pseudorecombinants with ICMV, and unique biological characteristics of DNA A. Although SLCMV is less closely related to ACMV than ICMV in overall nucleotide sequence identity, it can produce a viable pseudorecombinant with ACMV in *N. benthamiana.* The production of viable pseudorecombinants by reassortment of genomic components is generally restricted to strains of a particular virus (Stanley *et al.,* 1985; Lazarowitz, 1991; von Arnim and Stanley, 1992; Frischmuth *et al.,* 1993). The combination ACMV DNA A and SLCMV DNA B produces a systemic infection in this host to give symptoms resembling a mild infection by either parent. The mild phenotype indicates that the reassorted components are less well adapted to each other than the parental components. We have been unable to demonstrate that the reciprocal reassortment, SLCMV DNA A and ACMV DNA B, is able to produce a viable pseudorecombinant because of the ability of SLCMV DNA A to cause an aggressive systemic infection of *N. benthamiana* in the absence of DNA B. However, our observation that SLCMV DNA A is able to transreplicate ACMV DNA B in *N. benthamiana* leaf disks, coupled with the fact that the DNA B-encoded gene products of some Old World begomoviruses are functionally interchangeable (Frischmuth *et al.,* 1993), suggests that a viable pseudorecombinant could be produced under appropriate conditions. It is likely that SLCMV and ACMV can produce a viable pseudorecombinant because they have the identical iteron sequence AATTGGAGACA in their intergenic regions, facilitating Rep binding to initiate rolling circle replication (Argüello-Astorga *et al.,* 1994; Fontes *et al.,* 1994a). The ICMV iterons differ, explaining the replicational incompatibility with both SLCMV and ACMV. Intervening sequences between the iterons and the stem-loop also contribute to Rep binding specificity (Fontes *et al.,* 1994b), and differences between SLCMV and ACMV in this region, as well as the presence of a third cryptic iteron in the ACMV sequence, may contribute to the less aggressive nature of the pseudorecombinant.

SLCMV DNA A has characteristics of a monopartite begomovirus. In the absence of DNA B, it can induce upward leaf roll and vein swelling symptoms in *N. benthamiana* that are identical to those caused by monopartite begomoviruses such as AYVV (Tan *et al.,* 1995). Interestingly, these symptoms are also indistinguishable from those produced in this host by the monopartite beet curly top virus (BCTV, genus *Curtovirus*). This suggests that SLCMV gene *AC4,* similar to that of BCTV (Stanley and Latham, 1992; Latham *et al.,* 1997) but unlike its counterparts in other bipartite begomoviruses (Etessami *et al.,* 1991; Pooma and Petty, 1996), may play an important role in the phenotype. In addition, we have demonstrated that SLCMV DNA A can functionally interact with the satellite DNA β component associated with yellow vein disease of *A. conyzoides* (Saunders *et al.,* 2000). To date, DNA β components have only been found in association with monopartite begomoviruses such as AYVV, CLCuV, honeysuckle yellow vein mosaic virus (HYVMV),

and eupatorium yellow vein virus (EupYVV) (Saunders *et al.,* 2000; Briddon *et al.,* 2001; K. Saunders and J. Stanley, unpublished data). Although our data indicate that the bipartite begomoviruses ACMV and ICMV are able to transreplicate DNA β, their DNA A components are unable to functionally interact with the satellite DNA to produce a symptomatic systemic infection. Leaf curl and vein greening symptoms produced in *N. glutinosa* by SLCMV DNA A and DNA β are slightly milder than those produced by AYVV DNA A and DNA β but otherwise are identical. Significantly, the interaction with DNA β has been shown to extend the host range of SLCMV DNA A to include *A. conyzoides*. Symptoms induced in this host resemble those associated with AYVV DNA A and DNA β, demonstrating that SLCMV DNA A has the potential to behave in the same way as a monopartite begomovirus.

Nucleotide sequence comparisons clearly demonstrate that SLCMV DNA B is a recombinant. Although DNA A components of bipartite begomoviruses are generally more highly conserved than DNA B components, this is not the case for SLCMV and ICMV. SLCMV and ICMV DNA B sequences are extremely similar outside of the common region but show greatly reduced homology between their common region sequences, particularly those upstream of the stem-loop. This region is highly conserved between SLCMV components, suggesting that SLCMV DNA B has arisen by a recombination event between SLCMV DNA A and ICMV DNA B. We have previously demonstrated that ACMV DNA A will readily donate intergenic region sequences to a DNA B replication incompetent mutant *in planta* in order to restore infectivity (Roberts and Stanley, 1994) and our current observations have shown that a similar recombination event can occur between distinct species, on this occasion to produce the reciprocal viable pseudorecombinant between ICMV DNA A and SLCMV DNA B. That the SLCMV DNA A sequences downstream of the stem-loop region are more closely related to ICMV DNA B than SLCMV DNA B suggests that the nick site within the origin of replication, known to be a recombinational hotspot, was involved in this process. The fact that SLCMV DNA B is a recombinant does not rule out the possible exchange of genetic information between coinfecting bipartite begomoviruses. However, in view of the biological characteristics of SLCMV discussed above, the data are consistent with our suggestion that SLCMV DNA A has evolved from a monopartite begomovirus by capturing the DNA B component from a bipartite begomovirus. The fact that the DNA B sequences external to the common region remain highly conserved between SLCMV and ICMV suggests that this was a relatively recent event. This implies that a monopartite begomovirus progenitor of SLCMV DNA A has existed (or still exists), possibly in association with a satellite DNA β component. A detailed survey is necessary to establish whether such a virus occurs in Sri Lanka, India, or elsewhere and, if so, in which host(s) it is maintained. Attempts to PCR-amplify a DNA β component from scDNA isolated from SLCMV-infected plants using generic primers and conditions that have been used to amplify similar components from extracts of plants infected with AYVV, HYVMV, and EupYVV have been unsuccessful, suggesting that the SLCMV isolate is not associated with either a full-length DNA β component or a remnant thereof as occurs in ToLCV-infected tissues (Dry *et al.,* 1997; Saunders *et al.,* 2000). To date, the only other begomovirus reported from Sri Lanka was isolated from tomato (Accession No. AF274349) and only a DNA A sequence is available. Whether or not it is a monopartite begomovirus remains to be established, but it is distinct from SLCMV DNA A (73.7% nucleotide identity).

The impact of begomovirus diseases has drastically increased in recent years as a result of changes in agricultural practices and dispersal of whitefly vector biotypes that have provided new opportunities for the viruses to adapt to different hosts and environmental conditions. The much wider dissemination of begomoviruses provides greater opportunity for mixed infections, allowing recombination to play an important role in begomovirus diversity (Padidam *et al.,* 1999). For example, recombination between cassava-infecting bipartite begomoviruses was probably responsible for the severe outbreak of CMD in Uganda (Zhou *et al.,* 1997; Fondong *et al.,* 2000; Pita *et al.,* 2001), and it has been suggested that the emergence of cotton leaf curl disease in Pakistan was the result of recombination between endemic monopartite and bipartite begomoviruses (Sanz *et al.,* 2000). Our present results provide evidence that a recombination event has allowed a monopartite begomovirus to rapidly evolve into a bipartite begomovirus simply by donating intergenic sequences involved in replication. In view of the importance of DNA B genes to virus pathogenicity and host adaptation (Ingham *et al.,* 1995; Schaffer *et al.,* 1995), it is likely that the capture of a second component in this way would lead to an alteration in host range, providing additional opportunities for the recombinant virus to exploit. An equally intriguing possibility is that a similar recombination event could allow a bipartite begomovirus to exchange its DNA B component for a satellite DNA β component, thereby producing a monopartite begomovirus. While this has not yet been observed, we have recently demonstrated that such a recombination event with AYVV DNA A has occurred naturally to produce a viable DNA β recombinant (Saunders *et al.,* 2001).

MATERIALS AND METHODS

Virus source and maintenance

Cuttings taken from CMD-affected cassava cultivar H-165 growing in Maharashtra state in 1988 were maintained in an insect-free glasshouse at the Marathwada

Agricultural University, Parbhani, India. Infected leaf tissue was collected and transported to the John Innes Centre for virus isolation and characterisation. CMD-affected cassava of an undefined cultivar originating from Columbo, Sri Lanka, was transported to the John Innes Centre in 1998. Plants were maintained in an insect-proof glasshouse at 25°C (reduced to 20°C at night) with supplementary lighting to give a 16-h photoperiod. Virus was transmitted from cassava to *N. benthamiana* by mechanical inoculation.

Construction of infectious recombinant DNA clones

Nucleic acids were extracted from systemically infected *N. benthamiana* tissue according to the method of Etessami *et al.* (1991), and viral scDNA was purified by two cycles of CsCl gradient centrifugation as described by Stanley *et al.* (1985). Full-length copies of the genomic components of ICMV (isolate NB1) were inserted into pIC20R (Marsh *et al.,* 1984) after linearisation of the scDNA using *Pst*I (DNA A clones pICM8–pICM11), *Hin*dIII (DNA A clones pICM17–pICM22), and *Bam*HI (DNA B clones pICM12–pICM16). Full-length copies of the genomic components of SLCMV (isolate CV2) were inserted into pBluescript SK+ (Stratagene) after linearisation of the scDNA using *Hin*dIII (DNA A clones pSL1 and pSL6, and DNA B clones pSL3, pSL4, and pSL9). The sequences of both strands of ICMV DNA A (pICM18) and DNA B (pICM15), and SLCMV DNA A (pSL1) and DNA B (pSL4), were determined using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer) with primers specific to either the cloning vectors or the established viral sequences. Sequencing products were resolved using an ABI 373 automated sequencer. Sequences were analysed using version 8 of the program library of the Genetics Computer Group (Devereux *et al.,* 1984).

For the purpose of agroinoculation, the genomic components were subcloned as partial repeats into *A. tumefaciens* binary vectors. The *Hin*dIII(79)–*Eco*RI(1664) fragment of ICMV DNA A (pICM18) was cloned into pBin19 (Bevan, 1984), and the full-length *Hin*dIII fragment was then inserted to produce pBinICM1.6A. The *Bam*HI(1427)–*Bgl*II(321) and *Bgl*II(321)–*Bam*HI(1427) fragments of ICMV DNA B (pICM15) were cloned into pBin19, and the full-length *Bam*HI fragment was then inserted to produce clones pBinICM1.6B and pBinICM1.4B, respectively. SLCMV DNA A clone pSL1 was digested with *Pst*I to remove the *Pst*I(1433)–*Hin*dIII(2499) fragment and adjacent pBluescript SK+ polylinker sequences. The full-length *Hin*dIII fragment was then inserted to produce pSLCM1.6A. SLCMV DNA B clone pSL4 was digested with *Eco*RI to remove the *Hin*dIII(1901)–*Eco*RI(2465) fragment and adjacent pBluescript SK+ polylinker sequences. The full-length *Hin*dIII fragment was then inserted to produce pSLCM1.8B. The pSLCM1.6A and pSLCM1.8B inserts were subcloned into pBinPlus (van Engelen *et al.,* 1995) as *Sac*I/*Sal*I fragments to produce pBinSLCM1.6A and pBinSLCM1.8B, respectively. The construction of a partial repeat of ACMV DNA A and a tandem repeat of ACMV DNA B in pBin19 (pBin1.3A and pBin2B) has been described by Klinkenberg *et al.* (1989). The construction of a partial repeat of AYVV DNA A in pBin19 (pBinAYVVA), a tandem repeat of AYVV DNA β in pBinPlus (pBinAYVVβ), and their cloning together on the same binary vector (pBinAYVVAβ) has been described by Saunders *et al.* (2000, 2001).

Whole plant inoculation and viral DNA replication assays using cloned viral components

In preliminary experiments, the inserts of ICMV clones pICM15 and pICM18 (5 μg per plant) and SLCMV clones pSL1 and pSL4 (1 μg per plant) were mechanically inoculated onto *N. benthamiana* plants. Subsequently, *Nicotiana* spp. and *D. stramonium* were agroinoculated in the stem when 2–3 weeks old as described by Tan *et al.* (1995) using *A. tumefaciens* strain GV3850 (Zambryski *et al.,* 1983) transformed with the binary vector constructs described above. Cassava plants were inoculated biolistically with excised inserts of SLCMV clones pSL1 and pSL6 as described by Briddon *et al.* (1998). Viral DNA replication was assayed by agroinoculating *N. benthamiana* leaf disks using *A. tumefaciens* strain LBA4404 (Hoekema *et al.,* 1983) as described by Klinkenberg *et al.* (1989). Leaf disks were harvested 2, 4, and 6 days post-inoculation.

Analysis of viral DNAs

Nucleic acids were isolated from systemically infected plant material using the procedure of Covey and Hull (1981) and were fractionated by agarose gel electrophoresis in TNE buffer (40 mM Tris–acetate, pH 7.5, 20 mM Na acetate, 2 mM EDTA) and transferred to Hybond-N (Amersham Life Science) as described by Saunders and Stanley (1999). Dot-blot analyses were performed as described by Maule *et al.* (1983). Viral DNAs were detected using probes to ACMV DNA A (*Nhe*I(1062)–*Eco*RI(1716); *Nhe*I site introduced by mutagenesis) and DNA B (*Pst*I(93)–*Eco*RV(2398)), ICMV DNA A (*Hin*dIII(79)–*Kpn*I(1574)) and DNA B (*Hin*dIII(141)–*Bam*HI(1427)), SLCMV DNA A (*Sph*I(1343)–*Hin*dIII(2499)) and DNA B (*Eco*RV(207)–*Hin*dIII(1901)), and AYVV DNA A and DNA β (Saunders *et al.,* 2001). Fragments were oligolabelled as described by Feinberg and Vogelstein (1983).

To investigate possible recombination events, a fragment of SLCMV DNA B was PCR-amplified from isolated nucleic acids using the virion-sense primer V4503 (corresponding to nucleotides 1953–1971) and complementary-sense primer V4509 (corresponding to nucleotides 830–852). Similarly, a fragment of AYVV DNA β was

amplified using the virion-sense primer V3996 (corresponding to nucleotides 795–821) and complementary-sense primer V3518 (corresponding to nucleotides 286–310). Fragments were amplified from 100-ng samples using 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s. Amplified fragments were purified by a Wizard PCR Preps DNA purification system (Promega) and cloned into pGEM-T Easy (Promega), and selected clones were sequenced.

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