

Phylogenetic analysis and biolistic infectivity of a cloned Sri Lankan cassava mosaic virus DNA-A from Tamil Nadu, India on *Nicotiana benthamiana*

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Summary. – The complete nucleotide sequence of Sri Lankan cassava mosaic virus (SLCMV) DNA-A isolated from cassava in southern India was analyzed, a phylogenetic analysis with other begomoviral nucleotide sequences was performed and an efficient inoculation method of *Nicotiana benthamiana* with the cloned DNA was developed utilizing a biolistic device. The nucleotide sequence showed the conservation of all functional begomoviral protein domains and aligned most closely with begomoviruses reported from the Indian subcontinent, except for begomoviruses of cucurbits and legumes. Upon biolistic inoculation of *N. benthamiana* with the cloned DNA, most plants became symptomatic and showed the accumulation of viral DNA within 21–28 days post-inoculation.

Keywords: cassava; begomovirus; rolling circle amplification; dendrogram; biolistic inoculation

Introduction

Geminiviruses (the family *Geminiviridae*) cause significant yield losses in crop plants throughout the tropical and sub-tropical parts of the world (Thresh *et al.*, 1997; Patil and Fauquet, 2009; Castillo *et al.*, 2011). Members of the genus *Begomovirus* (the family *Geminiviridae*) are transmitted by whiteflies of the species *Bemisia tabaci* (Sanderfoot and Lazarowitz, 1996) and consist of one (monopartite) or two (bipartite) circular single-stranded DNA components of about 2.7 kb in size. DNA-A components encode proteins necessary, among others, in replication and encapsidation; whereas DNA-B encodes proteins with functions related to the movement of viral DNA within and between the host cell (Kheyr-Pour *et al.*, 1991; Fauquet *et al.*, 2008). There is

an approximately 200 bp common region (CR) within the intergenic region of both DNA components, which share high similarity with each other (Harrison and Robinson, 1999). The common region is important for the initiation of DNA replication. The proteins encoded by DNA-A include the coat protein (AV1), the replication-associated protein AC1 (Rep), and the remaining proteins that have roles as a pathogenicity determinant (AC2), enhancer of viral DNA accumulation (AC3) and symptom determinant (AC4) in various begomoviruses (Morris *et al.*, 1991; Azzam *et al.*, 1994; Jupin *et al.*, 1995; Sung and Coutts, 1995; Vanitharani *et al.*, 2004). AC2 and AC4 have also been reported to act as suppressors of RNAi, a defence response of plants against viruses, thus contributing towards the viral virulence functions (Vanitharani *et al.*, 2004).

Cassava mosaic disease (CMD) is widespread in cassava (*Manihot esculenta* Crantz, the family *Euphorbiaceae*) in the African continent, India and Sri Lanka (Abraham, 1956, Alagianagalingam and Ramakrishnan, 1966). Two bipartite begomoviruses, Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV) are associated with CMD in India (Saunders *et al.*, 2002; Dutt *et al.*, 2005;

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Abbreviations: CMD = cassava mosaic disease; psi = pounds per square inch; RCA = rolling circle amplification; SLCMV = Sri Lankan cassava mosaic virus

Patil *et al.*, 2005). SLCMV-Adi, an isolate from southwestern India (Adivaram, Kerala) has been analyzed earlier, focusing mainly on the CR and the infectivity of the DNA components using agro-inoculation, a method of introduction of the viral DNA into plants using *Agrobacterium*-mediated inoculation of viral DNA cloned as a partial dimer (Grimsley *et al.*, 1986; Dutt *et al.*, 2005). In this study, we have analyzed the DNA-A component of an SLCMV isolate obtained from Attur, Tamil Nadu, India, for the presence of known functional domains of begomoviral proteins. We have compared the sequence with other begomoviral sequences available in the databases from India and elsewhere to study its phylogenetic relationship and have standardized the procedure for biolistic inoculation of the laboratory host *N. benthamiana*, in which DNA molecules are inoculated to plants using pressurized metal microcarriers (Kikkert *et al.*, 2004),

Materials and Methods

Genomic DNA isolation: Symptomatic cassava stem cuttings were collected in the year 2009 from field locations near Attur, district Salem, Tamil Nadu, India. The cuttings were planted in greenhouses of University of Delhi South Campus, New Delhi and fresh leaves, sprouting from the above cuttings were used for the extraction of total genomic DNA using SDS method (Dellaporta *et al.*, 1983). Symptomatic leaves of *N. benthamiana*, following biolistic inoculation were used to isolate DNA using SDS method.

Rolling circle amplification (RCA) of total DNA extracted from infected plants. Viral DNA was amplified by Rolling circle amplification (RCA) using the TempliPhi™ Amplification Kit (GE Healthcare, Amersham) as per manufacturer's recommendation.

Cloning and sequencing. Digestion with *Pst*I was performed to linearize the RCA product and the resulting 2.7 kb fragment was cloned in pTZ57R vector (MBI Fermentas) and completely sequenced using a primer walking strategy (Haible *et al.*, 2006; Homs *et al.*, 2008). DNA sequencing was performed at the University of Delhi South Campus sequencing facility.

Sequence analysis. Begomoviral genes and their predicted amino acid sequences were searched by BLAST at NCBI server (www.ncbi.nlm.nih.gov/) and the software Gene Runner version 3.05. Nucleotide identities between cloned viral DNA molecule and other selected begomoviruses were analysed by the ClustalW method and NCBI BLAST server (Thompson *et al.*, 1994).

Phylogenetic analysis. A phylogenetic tree (1000 bootstrap replications) was constructed of the cloned viral DNA and sequences of other begomoviral DNA-A's, downloaded from nucleic acid sequence databases, by using the Neighbour-joining method in MEGA4.0 software (Tamura *et al.*, 2007). GenBank Acc. Nos. of DNA-A of various begomoviruses used for sequence analysis and for the construction of the phylogenetic tree are given in Table 1.

Southern blot analysis: Genomic DNA isolated from the leaves of plants biolistically inoculated with the SLCMV DNA-A were size

separated by gel electrophoresis, transferred to a nylon membrane and hybridized using standard methods (Sambrook and Russel, 2001). DNA fragments were labeled using Amersham™ Megaprime DNA Labeling System (GE Healthcare) kit and used as probe.

Infectivity analysis. For infectivity analysis, the viral insert (2.7 kb) was released from the vector backbone by digesting with *Pst*I, self-ligated, amplified by RCA and used for biolistic inoculation by Bio-Rad hand-held biolistic device (Catalog No. 165–2431 and 165–2432). The distance between leaf tissue and biolistic device was kept at 2 cm. *N. benthamiana* plants were grown for 30 days at 28°C and 12 hr photoperiod and inoculated at the third or fourth true leaf stage. Viral replication in inoculated plants was verified by Southern blot analysis. A DNA fragment representing the AV1 of the viral clone was used as a radioactive probe.

Results and Discussion

General feature of begomoviruses associated with CMD

DNA sequence analysis of the cloned viral DNA revealed 2758 nucleotides, which shared 99% identity with SLCMV-Adivaram DNA-A [AJ579307]. Hence the virus was considered to be an isolate of SLCMV and was given the descriptor SLCMV-IN[IN:Attur:09], (SLCMV-Attur, GenBank Acc. No. KC424490), named after Attur, the nearest town from the site of collection of the isolate. In SLCMV-Attur DNA-A, there were seven ORFs; two in the virion-sense (AV1 & AV2), and five in the complementary-sense orientation (AC1-AC5), typical of Old World begomoviruses. The nucleotide coordinates of AV1 and AV2 were 297–1067 and 137–493, encoding proteins of predicted 256 (29.8 kDa) and 118 amino acid residues (13.6 kDa), respectively. The nucleotide coordinates of AC1, AC2, AC3, AC4, and AC5 were 1546–2601, 1209–1616, 1064–1468, 2145–2447, and 327–812, respectively, encoding proteins with predicted 352 (39.5 kDa), 135 (15.0 kDa), 134 (15.8 kDa), 100 (10.8 kDa), and 161 (18.02 kDa) amino acid residues.

Motif analysis

All functional domains identified in the AV1 protein homologs in other begomoviruses were present in SLCMV-Attur at comparable positions, for example ¹⁶KVRRR²⁰ and ⁵¹RK⁵² (nuclear localization, Sigrid *et al.*, 2001). Domains ¹⁴FLTYP¹⁸ (binding to dsDNA), ⁵⁶HLH⁵⁸ (metal-binding site that may be involved in protein conformation and DNA cleavage), ¹⁰⁰DVKXYXXKD¹⁰⁸ (DNA cleavage, Beverly and Hanley-Bowdoin, 1998) and ²²⁰GDSRTGKT²²⁷ (ATP binding, Desbiez *et al.*, 1995), found in AC1 were also present. Similarly for AC2, ²⁹RRRR³² (nuclear localization signal), C³⁷, C³⁹, H⁴⁴, H⁵⁴, and C⁴⁷ (promoter activation and silencing suppression) were all present in SLCMV-Attur (Daniela *et al.*, 2005; Hartitz *et al.*, 1999).

Table 1. Overall sequence identities of 44 geminiviral DNA-A sequences from Indian subcontinent and elsewhere with the DNA-A of SLCMV-Attur

Full name	Abbreviation name	EMBL Acc. No.	Percentage nucleotide identity
Bean golden yellow mosaic virus – [Puerto Rico-Japan]	BGYMV-PR:JR	D00201.1	65
Bhendi yellow vein mosaic virus – India[India;Madurai]	BYVMV-IN[IN:Mad]	AF241479.1	76
Bhendi yellow vein mosaic virus – Pakistan[Pakistan:Multan301:1996]	BYVMV-PK[PK:M301:96]	AJ002453.1	76
Cowpea golden mosaic virus – [Nigeria:Nsukka:1990]	CGMV-[NG:Nsu:90]	AF029217.1	68
Chilli leaf curl virus isolate Taq4 – Oman[Oman:2005]	ChiLCV-OM[OM:05]	JN604495.1	77
Cotton leaf curl Bangalore virus [India:Bangalore:OY136A:2005]	CoLCBV-IN[IN:Ban:05]	GU112003.1	77
Cotton leaf curl Multan virus – Hisar[India:New Delhi:1999]	CLCuMV-His[IN:ND:99]	AY765256.1	76
Cotton leaf curl Multan virus – Hisar [Pakistan:Faisalabad 3]	CLCuMV-His[PK:Fai3]	AJ132430.1	76
Cucurbit leaf crumple virus – [United States of America:Arizona:1991]	CuLCrV-[US:Ari:91]	AF256200.4	66
East African cassava mosaic Kenya virus – [Kenya:Kathiana:K300:2002]	EACMKV-[KE:Kat:K300:02]	AJ717580.1	73
East African cassava mosaic Zanzibar virus – [Kenya:Felunzi:K19:2001]	EACMZV-[KE:Fel:K19:01]	AJ717562.1	73
Horsegram yellow mosaic virus – [India:Bangalore:French bean:2004]	HgYMV-[IN:Ban:FB:04]	AM932425.1	67
Indian cassava mosaic virus – India [India:Maharashtra:1988]	ICMV-IN[IN:Mah:88]	AJ314739.1	85
Jatropha yellow mosaic India virus	JYMIV-IN	FJ177030.2	76
Kudzu mosaic virus – [Vietnam:Hoabinh:2005]	KuMV-[VN:Ho:05]	DQ641690.1	68
Mungbean yellow mosaic virus – [Pakistan:14]	MYMIV-[PK:14]	AJ512495.1	66
Malvastrum leaf curl virus – [China:Guangxi 87:2004]	MaLCV-[CN:Gx87:04]	AJ971263.1	74
Mesta yellow vein mosaic Bahraich virus – [India:Bhanga:2008]	MEYVMBV-IN[IN:Bha:08]	FJ159268.1	76
Mungbean yellow mosaic India virus Indonesia isolate Bogor	MuYMI-V-[ID:11]	JN368432.1	67
Mungbean yellow mosaic India virus – [India:Varanasi:Dolichos]	MYMIV-[IN:Var:Dol]	AY547317.1	66
Mungbean yellow mosaic India virus – [Pakistan:106]	MYMIV-[PK:106]	AJ512498.1	66
Okra leaf curl virus – [Mali]	OLCV-[ML:07]	EU024119.1	73
Okra yellow mosaic Mexico virus – [Mexico:Mazatepec 3:2004]	OYMMV-[MX:Maz3:04]	DQ022611.1	68
Papaya leaf curl virus isolate Lucknow	PaLCuV-IN[IN:Luc]	JN135233.1	77
Papaya leaf curl virus – Pakistan[Pakistan:Cotton:2002]	PaLCuV-PK[PK:Cot:02]	AJ436992.1	76
Rhynchosia yellow mosaic virus – [Pakistan:Lahore]	RYMV-[PK:Lah:09]	AM999981.1	68
South African cassava mosaic virus – [Zimbabwe:Muzarabani]	SACMV-[ZW:Muz]	AJ575560.1	74
Sida leaf curl virus – [China:Hainan 57:2004]	SiLCuV-[CN:Hn57:04]	AM050730.1	73
Sida yellow vein Madurai virus – [India:Madurai:2005]	SiYV-MaV-[IN:Mad:05]	AM259382.1	74
Sri Lankan cassava mosaic virus – India[India:Adivaram]	SLCMV-IN[IN:Adi]	AJ579307.1	99
Sri Lankan cassava mosaic virus – Sri Lanka [Sri Lanka:Colombo:1998]	SLCMV-LK[LK:Col:98]	AJ314737.1	93
Squash leaf curl China virus – India [India:Coimbatore:Pumpkin]	SLCCNV-IN[IN:Coi:Pum]	AY184487.1	73
Squash leaf curl virus isolate YL segment DNA A [Taiwan:Yunlin]	SLCCV-TW[TW:Yun]	EU479710.1	74
Sweet potato leaf curl Bengal virus – [India:West Bengal:2008]	SWPLCBV-IN[IN:WB:08]	FN432356.2	64
Tomato leaf curl Bangladesh virus – [Bangladesh:2]	ToLCBDV-[BD:2]	AF188481.1	77
Tomato leaf curl Bangalore virus – [India:Kerala IV:2005]	ToLCBV-IN[IN:Ker:05]	DQ887537.1	75
Tomato leaf curl Pakistan virus isolate Rahim Yaar Khan 1 clone PT7	ToLCPV-[PK:RYK:06]	DQ116884.1	79
Tomato leaf curl Sinaloa virus – [Nicaragua:Santa Lucia]	ToLCSinV-[NI:SL]	AJ608286.1	70
Tomato leaf curl Philippines virus isolate P36 [Philippines: Benguet:08]	ToLCuPV-[PH:Ben:08]	EU487029.2	72
Tomato yellow leaf curl Thailand virus – A [Thailand:2]	ToYLCTHV-[TH:2]	AF141922.1	70
Tomato yellow leaf curl virus – Israel [Israel:Rehovot:1986]	TYLCV-IL[IL:Reo:86]	X15656	70
Tomato yellow leaf curl virus – Iran [Iran:Iranshahr:1998]	TYLCV-IR[IR:Ira:86]	AJ132711.1	73
Tomato yellow leaf curl virus – Mild [Spain:72:1997]	TYLCV-Mld-[ES72:97]	AF071228.1	75
Velvet bean severe mosaic virus-Lucknow	VBSMV-IN[IN:UP:Luc]	FN543425.1	66

Phylogenetic analysis

Phylogenetic analysis revealed that SLCMV-Attur shared 64–99 percent sequence identities with 44 other begomoviral DNA-A sequences (Table 1) and was closely related to begomoviruses infecting plants from the families

Caricaceae, Euphorbiaceae, Malvaceae and Solanaceae from Indian subcontinent (Fig. 1). SLCMV-Attur had a distant relationship with legume-infecting (*Olufemi et al.*, 2010) and cucurbit-infecting begomoviruses from the Indian subcontinent and most begomoviruses from outside the Indian subcontinent.

Families of the primary hosts:

- I = Caricaceae
 II = Solanaceae
 III = Malvaceae
 IV = Euphorbiaceae
 V = Cucurbitaceae
 VI = Leguminosae
 VII = Convolvulaceae
 A = Indian Subcontinent
 B = Outside Indian subcontinent

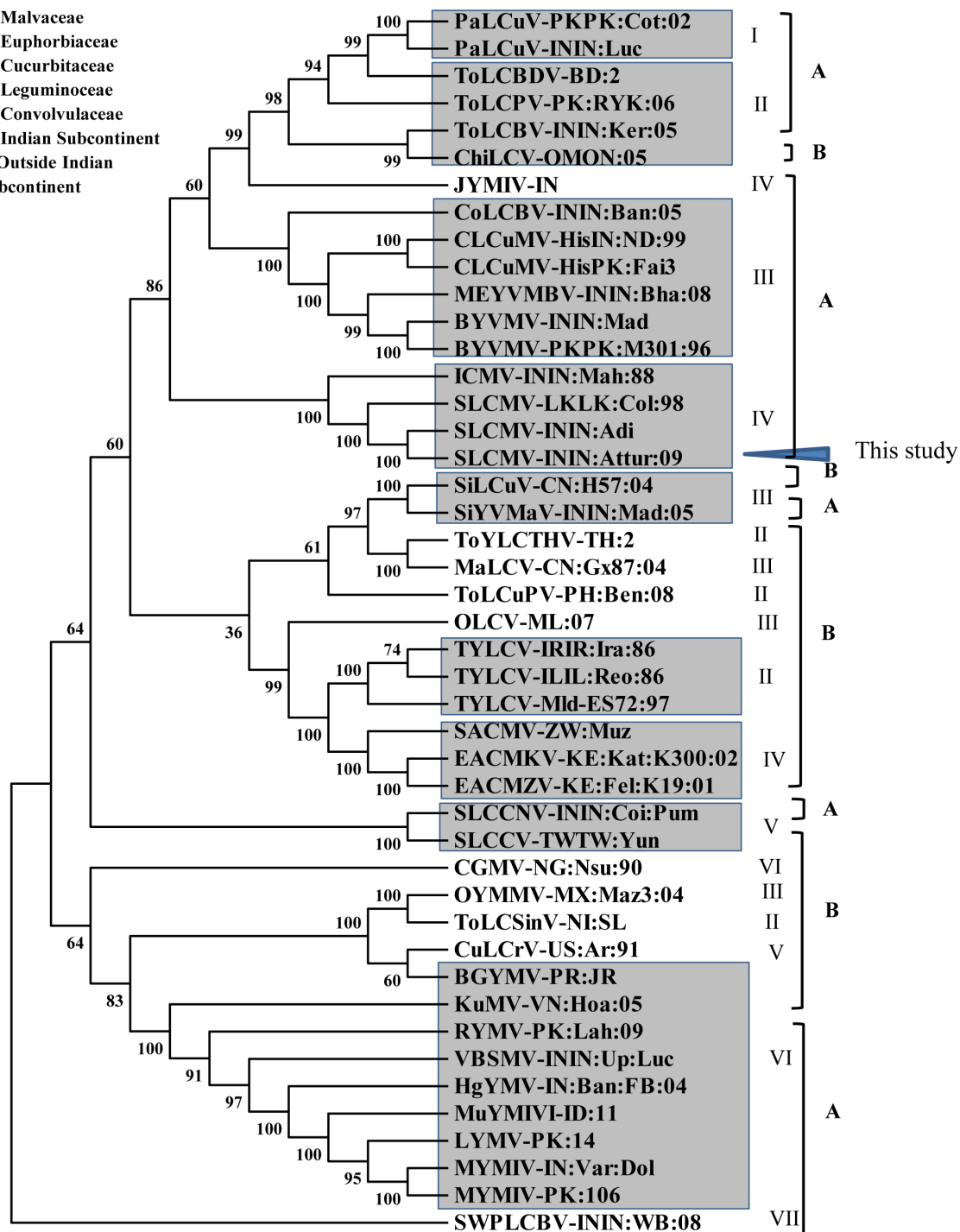


Fig. 1

Dendrogram showing the phylogeny of the complete nucleotide sequence of SLCMV-Attur DNA-A (shown in arrow) with begomoviral nucleotide sequences reported from the Indian subcontinent and from the outside of the Indian subcontinent

Horizontal distances are proportional to the sequence distances and vertical distances are arbitrary. A bootstrap analysis with 1,000 replicates was performed. The Families, to which the primary hosts belong, are indicated for each virus. Virus abbreviations are the same as in Table 1.

Infectivity of cloned component of SLCMV by Biolistic inoculation

When *N. benthamiana* plants were inoculated with 1000 ng of RCA-amplified circularized SLCMV-Attur DNA-A using the biolistic device, 56 out of 66 plants showed stunting and downward leaf curling between 21 to 28 days post-inoculation, when the DNA was inoculated under the pressure of 200 pounds per square inch (psi) (Fig. 2, Table 2). With the same DNA, at the pressure of 150 psi, the symptoms were mild downward leaf curling and no symptoms appeared at the pressure of 100 psi (Table 2). Eight inoculated plants showing severe symptoms were analyzed for the accumulation of SLCMV DNA by Southern blot. Seven of them gave the appropriate signals indicating viral DNA accumulation (Fig. 3). Begomoviral DNA molecules from three such plants were cloned and sequenced. The sequence (around 700 bp nucleotides) matched perfectly with SLCMV-Attur DNA-A. The symptoms were markedly different from the upward leaf rolling and vein swelling reported after infection with SLCMV-Adi via agroinoculation (Dutt *et al.*, 2005; Mittal *et al.*, 2009).

Cassava is a vegetatively propagated crop, and the close phylogenetic relationship between SLCMV-Attur and other SLCMV strains indicates that they have remained genetically uniform over time and across the regions where SLCMV thrives in India. This may be partly contributed to by the possible movement of infected cassava propagules. The low genetic identity of SLCMV-Attur with SLCMV-Colombo, an isolate reported from Sri Lanka, (Table 1) could be because of the geographical isolation of Sri Lanka from southern India and the lack of exchange of infected cassava propagules. The closer phylogenetic relationship of Indian begomoviruses with SLCMV-Attur than with those from outside the Indian subcontinent (Table 1, Fig. 1) points towards the involvement

Table 2. Infectivity of SLCMV-Attur DNA-A on *N. benthamiana* plants using three pressure conditions and two DNA quantities in biolistic inoculation after 21–28 days post-inoculation

Experiment No.	No. of symptomatic/ No. of inoculated plants	Pressure (psi)	Symptoms ^a	Amount of DNA (ng) used per plant
1	14/15	200	SD, DC	1000
2	13/15	200	SD, DC	1000
3	11/15	200	SD, DC	1000
4	12/15	200	SD, DC	1000
5	6/6	200	SD, DC	1000
6	5/15	150	MS, DC	1000
7	1/3	150	MS, DC	700
8	1/3	150	MS, DC	700
9	2/6	150	MS, DC	700
10	1/12	150	MS, DC	700
11	3/12	150	MS, DC	700
12	0/15	100	No symptoms	1000
13	0/12	200	No symptoms	0

^aSS = severe stunting, DC = downward leaf curling, MS = mild stunting.



Fig. 2

Symptoms produced by biolistic inoculation of *N. benthamiana* using SLCMV-Attur DNA-A, 30 days post-inoculation

Plant (a), control inoculation using only gold particles; plants (b-d), inoculation using SLCMV-Attur DNA-A. All three plants shown in (b-d) show severe stunting.

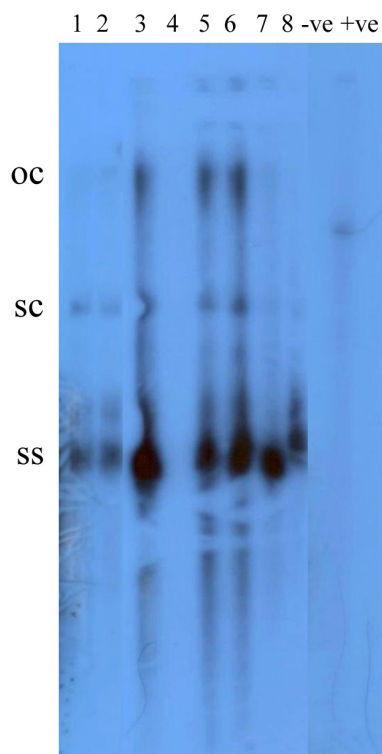


Fig. 3

Southern hybridization analysis showing replication of viral DNA in systemic leaf tissue of *N. benthamiana* plants biolistically inoculated with SLCMV-Attur DNA-A at 30 days post-inoculation

The blot was probed with a DNA fragment encoding the coat protein. Lanes 1-8 total DNA from leaves inoculated with SLCMV-Attur DNA-A, -ve, DNA from leaves inoculated with only microcarrier particles, +ve, cloned SLCMV DNA-A. Positions of open circular (ocDNA), super coiled (scDNA) and single stranded (ssDNA) viral DNA forms are indicated.

of the insect vector, the single polyphagous whitefly species, in the process of shaping the begomoviral genomes. The genetic isolation of begomoviruses from cucurbitaceous and leguminous plants is probably due to a host range barrier or lack of movement of whitefly vectors between these hosts and other plants, preventing the genetic exchange between these groups of viruses (Qazi *et al.*, 2007).

The downward leaf curling symptoms observed in the biolistic method, as opposed to upward leaf-rolling and vein swelling in agroinoculation, possibly results from DNA delivery into the epidermal or mesophyll cells, whereas the agroinoculation delivers it into the vasculature of the stem or the petiole, triggering different sets of interactions between the viral and host proteins or RNAs, thus giving rise to different symptoms. This hypothesis, however, needs deeper investigation.

An earlier report has described biolistic inoculation of cassava plants using SLCMV-Adi (Dutt *et al.*, 2005). The method of biolistic inoculation described in this paper gives more details on the conditions to be used to obtain optimal infections and can be used as the starting point for optimizing its infectivity on the natural host cassava.

Future perspective

Biolistic delivery of SLCMV DNA to *N. benthamiana* opens up the possibility of analysis of its gene functions by site-directed mutagenesis.

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