

# Studies on differential behavior of cassava mosaic geminivirus DNA components, symptom recovery patterns, and their siRNA profiles

Basavaprabhu L. Patil · Claude M. Fauquet

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**Abstract** Cassava mosaic disease caused by cassava mosaic geminiviruses (CMGs) with bipartite genome organization is a major constraint for production of cassava in the African continent and the Indian sub-continent. Currently, there are eleven recognized species of CMGs, and several diverse isolates represent them, with vast amount of sequence variability, reflecting into diversity of symptom severity/phenotypes. Here, we make a systematic effort to study the infection dynamics of several species of CMGs and their isolates. Further, we try to identify the genomic component of CMGs contributing to the manifestation of diverse patterns of symptoms and the molecular basis for the differential behavior of CMGs. The pseudo-recombination studies carried out by swapping of DNA-A and DNA-B components of the CMGs revealed that the DNA-B component significantly contributes to the symptom severity. Past studies had shown that the DNA-A component of Sri Lankan cassava mosaic virus shows

monopartite feature. Thus, the ability of DNA-A component alone, to replicate and move systemically in the host plant with inherent monopartite features was investigated for all the CMGs. Geminiviruses are known to trigger gene silencing and are also its target, resulting in recovery of the host plant from viral infection. In the collection of several different CMG species and isolates we had, there was a vast variability in their recovery and non-recovery phenotypes. To understand the molecular basis of this, the origin and distribution of virus-derived small interfering RNAs were mapped across their genome and across the CMG-infected symptomatic *Nicotiana benthamiana*.

**Keywords** Geminivirus · Begomovirus · Cassava mosaic virus · Symptom recovery · Pseudo-recombination · siRNA

## Introduction

Cassava mosaic disease (CMD) caused by cassava mosaic geminiviruses (CMGs) is an important disease of cassava (*Manihot esculenta* Crantz), in the African continent and the Indian sub-continent [1–3]. CMGs have bipartite genomes (DNA-A and DNA-B) typical of an Old World bipartite begomovirus, and both of the genomic components have a highly conserved intergenic common region (CR) encompassing a stem-loop structure with an invariant nonanucleotide sequence (TAATATTAC) [4]. The DNA-A component encodes for proteins required for replication (AC1) or its enhancement (AC2-AC4), and encapsidation (AV1) of the viral genome [2, 4], whereas the DNA-B component encodes for the nuclear shuttle protein (BV1) and the cell-to-cell movement protein (BC1) required for the movement of viral genome. Globally there are 11 recognized species of CMGs, of which nine are reported from

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B. L. Patil · C. M. Fauquet  
Danforth Plant Science Center, 975 N. Warson Rd., St. Louis,  
MO 63132, USA

B. L. Patil (✉)  
National Research Center on Plant Biotechnology, Pusa Campus,  
New Delhi, India  
e-mail: blpatil2046@gmail.com

*Present Address:*  
C. M. Fauquet  
Centro Internacional de Agricultura Tropical,  
Apartado Aéreo 6713, Cali, Colombia

Africa: *African cassava mosaic virus* (ACMV), *African cassava mosaic Burkina Faso virus* (ACMBFV), *Cassava mosaic Madagascar virus* (CMMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *East African cassava mosaic Kenya virus* (EACMKV), and *South African cassava mosaic virus* (SACMV), and two species from the Indian sub-continent: *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) [1, 2, 5]. Some of these species comprise several strains and isolates, which have evolved independently through recombination [2, 6]. Evolution of geminiviral sequences has led to the emergence of new genotypes exhibiting diverse infection patterns and host specificity [7]. Both recombination and pseudo-recombination events among geminiviruses have played an important role in CMG epidemiology and evolution by creating additional sources of biodiversity [2, 8]. In 1990s, a major epidemic broke out due to the emergence of a recombinant strain of EACMV referred as EACMV-Uganda in the cassava plantations of East Africa [8, 9]. Sequence analysis of geminiviruses from different parts of the world has revealed that the DNA-A and DNA-B components have evolved independently with distinct evolutionary history [10]. Past studies have shown that SLCMV evolved from a monopartite begomovirus to become a bipartite begomovirus by capturing the DNA-B component from ICMV [11]. Similarly the ACMV DNA-A component alone is capable of limited systemic spread in *Nicotiana benthamiana* in the absence of DNA-B component [12], although both the genomic components are required for production of visible symptoms. Monopartite begomoviruses, which mostly originated from the Old World, lack the DNA-B component, but are associated with satellite DNAs (betasatellites and/or alphasatellites) [10]. In the past, most of the infectivity studies and characterization of different species/isolates of CMGs were done independently. A comprehensive and comparative study involving all the CMG species and their strains/isolates was missing [13]. Geminiviruses are known to trigger gene silencing in host plants, which help the plants to recover from viral infections [14–16]. In the past, such symptom recovery over time was reported for ACMV and SLCMV which accumulate higher amounts of small interfering RNAs (siRNAs) in contrast to the non-recovering isolates of EACMCV and ICMV which accumulate lower levels of siRNA [17]. Fine mapping for accumulation of siRNAs across the genomes of CMGs demonstrated that the C-terminus of AC1 and N-terminus of AC2 were primary targets of gene silencing in ACMV and the C-terminus of BC1 was the primary target in EACMCV [17].

Here, we make a systematic effort to do a comparative study of the infection dynamics of most of the species of

CMGs and their isolates, which exhibit vast sequence diversity and also diverse patterns of symptoms, both in the laboratory host *N. benthamiana* and the natural host cassava. To understand the factors responsible for the manifestation of diverse symptom patterns produced by CMGs, we have done pseudo-recombination studies by swapping the DNA-A and DNA-B components, and we have also mapped the origin and distribution of viral siRNAs.

## Materials and methods

### Production of infectious CMGs and their infectivity studies

The monomeric clones of most of the EACMV-like begomovirus clones (EACMV-KE[KE:Kat:K24:01]; EACMV-KE[KE:Msa:K201:02]; EACMV-UG[KE:Wot:K282:02]; EACMKV[KE:Keh:K229:02]; EACMZV-[KE:Kib:K275:02] and EACMZV-[KE:Fel:K19:01]) were a generous gift of Dr. John Stanley (John Innes Center, Norwich, UK), which were used for developing infectious partial dimers [2, 6, 17]. To simplify the nomenclature, these viruses will be referred to as EACMV-K24, EACMV-K201, EACMV-UG, EACMKV-K229, EACMKV-K308, EACMZV-K275, and EACMZV-K19, respectively. These CMGs were cloned as tandem repeats in pBluescript II SK (+) and verified for their infectivity in *N. benthamiana* using PDS1000/He particle bombardment system (Bio-Rad Laboratories Inc., Hercules, CA, USA) and were also tested in cassava using Helios hand-held gene gun (Bio-Rad Laboratories Inc., Hercules, CA, USA) at a concentration of 200 ng of each viral DNA per plant [18]. Other EACMV-like virus isolates used as monomers in infectivity and DNA-B swapping experiments were EACMV-KE[KE:Mis:K27:01], EACMV-[KE:Kib:K29:01], EACMV-KE[KE:Boa:K48:01], EACMV-[KE:Mig:K268:02], EACMKV-[KE:Mat:K308:02], and EACMZV-[KE:Kwa:K3:01] [17]. These were released from their vector by digesting with *Sal* I for DNA-A component and *Sac* II for DNA-B component as discussed in Bull et al. [17] and inoculated by particle bombardment in *N. benthamiana* and by Bio-Rad's Helios hand-held gene gun in cassava.

Similarly the tandem repeat infectious clones of EACMCV-CM[CI:98] were sub-cloned into the binary vector (AKK 1420) for agro-inoculation studies, using the *Agrobacterium* strain GV3101 [19, 20]. Other agro-infectious CMG clones used in this study were ACMV-[KE:844:82] [21], SACMV-[ZA] [22], ICMV-IN[IN:Mah:88], and SLCMV-LK[LK:Col:98] [11]. In this manuscript, these CMGs will be referred as EACMCV-CI, ACMV-KE, SACMV-ZA, ICMV-Mah, and SLCMV-Col, respectively. Since ACMV-[KE:844:82] is not infectious in cassava [21], we used an infectious clone of another ACMV isolate ACMV-[CM:DO2:98] [19], which

produces a symptom phenotype like ACMV-KE in *N. benthamiana* but infects cassava unlike ACMV-KE. For all the infectivity studies in cassava, we used ACMV-[CM:DO2:98] inoculations by particle bombardment, which will be referred as ACMV-CM in this manuscript, whereas for all the agro-inoculation studies in *N. benthamiana*, the agro-infectious clone of ACMV-KE was used.

The symptom phenotypes obtained were similar for all the CMGs in *N. benthamiana* after using both the methods of virus inoculation (Particle bombardment and Agro-inoculation). However, most of the experiments described in this manuscript are based upon agro-inoculation of CMGs in *N. benthamiana*, since the *Agrobacterium*-mediated virus inoculation was more efficient, cost effective, and uniform among different replications, showing an early initiation of symptoms with increased severity. The method followed for generation of agro-infectious clones and their infectivity study is described in Patil and Fauquet [20]. The virus inoculated plants were maintained in controlled growth chambers with a constant temperature of 28 °C, 70 % RH, 200  $\mu\text{Em}^{-2}\text{s}^{-1}$  light, and 16 h light and 8 h dark period. Each combination of virus inoculation was done in ten *N. benthamiana* plants and six cassava plants for each experiment, and all the experiments were repeated four times in *N. benthamiana* and three times in cassava. The symptoms were scored on alternate days, for a period of 1 month, beginning from the initiation of symptoms [23]. The pictures of symptomatic plants shown/described in Figs. 1 and 2 represent the symptom phenotypes obtained for majority of plants for each virus combination. The virus symptoms were scored using a scale of 0–5 (0 indicates the absence of symptoms and 5 indicates the necrosis or death of the plant) [20, 23].

#### PCR detection of viral DNA, Southern and Northern hybridizations

PCR analyses were done for randomly selected plants, for all the combinations of inoculations, with primers designed to specifically amplify selected regions of DNA-A and DNA-B. Southern hybridizations were done at 1 and 3 weeks post-inoculation, for a fully opened third symptomatic leaf from top as described in Patil and Fauquet [20]. For detection of DNA-A, PCR amplification of DIG-labeled coat protein was used as probe, while for detection of DNA-B, the PCR amplification of movement protein was used as probe [20]. These probes were also used for Northern hybridizations for detection of viral transcripts [23].

#### Northern hybridizations for siRNA detection and reverse-Northern hybridizations

Small RNA was fractionated from the total RNA, using RNeasy plant mini kit (Qiagen) and the RNA cleanup

protocol to specifically elute the small RNA fraction as described in Patil et al. [24]. For siRNA detection, 10  $\mu\text{g}$  of small RNA was loaded onto Criterion pre-cast 15 % TBE urea gel (Bio-Rad Laboratories Inc., Hercules, CA, USA) and subjected to Northern hybridization with a hydrolyzed probe obtained by in vitro transcription of cloned CMG fragments using SP6/T7 Transcription Kit (Roche Applied Science, Indianapolis, IN, USA) [23, 24].

For reverse-northern hybridization, young symptomatic leaves were collected from 3 weeks post-inoculation of virus in *N. benthamiana* and were subjected to small RNA isolation. About 50  $\mu\text{g}$  of above small RNA was resolved in Criterion pre-cast 15 % TBE urea gel (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the small RNAs in the size range of 15–30 nt were eluted as described by Chellappan et al. [16]. The eluted small RNAs were subjected to 3' end labeling by using the DIG oligonucleotide 3'-end labeling kit (Roche Applied Science, Indianapolis, IN, USA) and used as probe to map the origin and distribution of siRNAs across the genomes of CMGs. The entire genome of CMGs was PCR amplified as segments of  $\sim 400$  nt, starting from the origin of replication, resulting in seven segments (A1 to A7) for DNA-A component and another seven segments (B1 to B7) for DNA-B component (Fig. 4; Table 1). These were resolved on 1 % agarose gel and transferred to Hybond N<sup>+</sup> membrane by capillary transfer using 20  $\times$  SSC, for reverse-Northern hybridization, by using the end-labeled small RNA as the probe. The signal intensities on the auto-radiogram were quantified by Image-J program and normalized with the amount of nucleic acid (PCR product) loaded before statistical analysis, as in certain cases, there was no equal loading, particularly in the case of EACMZV-K275. The small RNA in the size range of 15–30 nt was eluted from mock inoculated *N. benthamiana*, and its cross-hybridization with the PCR segments of CMGs was also checked. The graphical representation of average signal intensities obtained for each PCR fragment of CMGs indicating the density of siRNA originating from these regions is also shown for each CMG (Fig. 4). Mock inoculations were done and subjected to molecular analysis (data not shown).

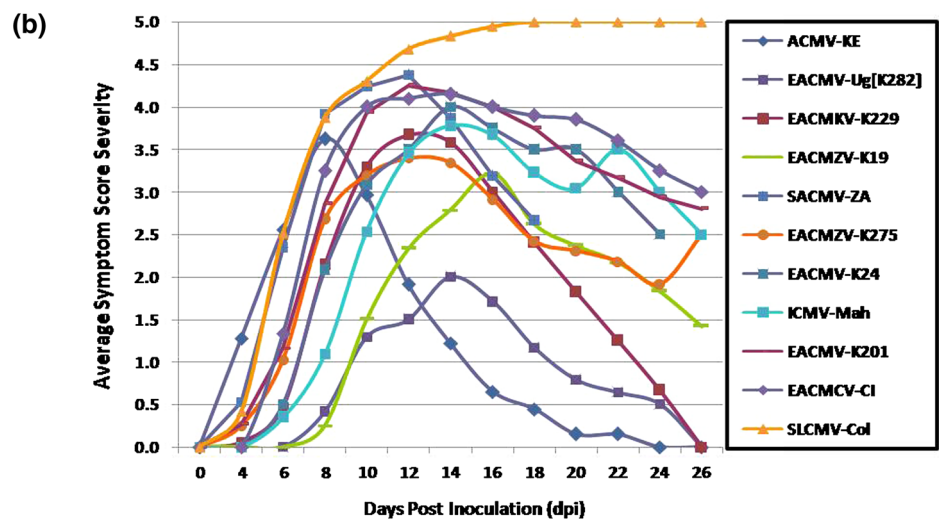
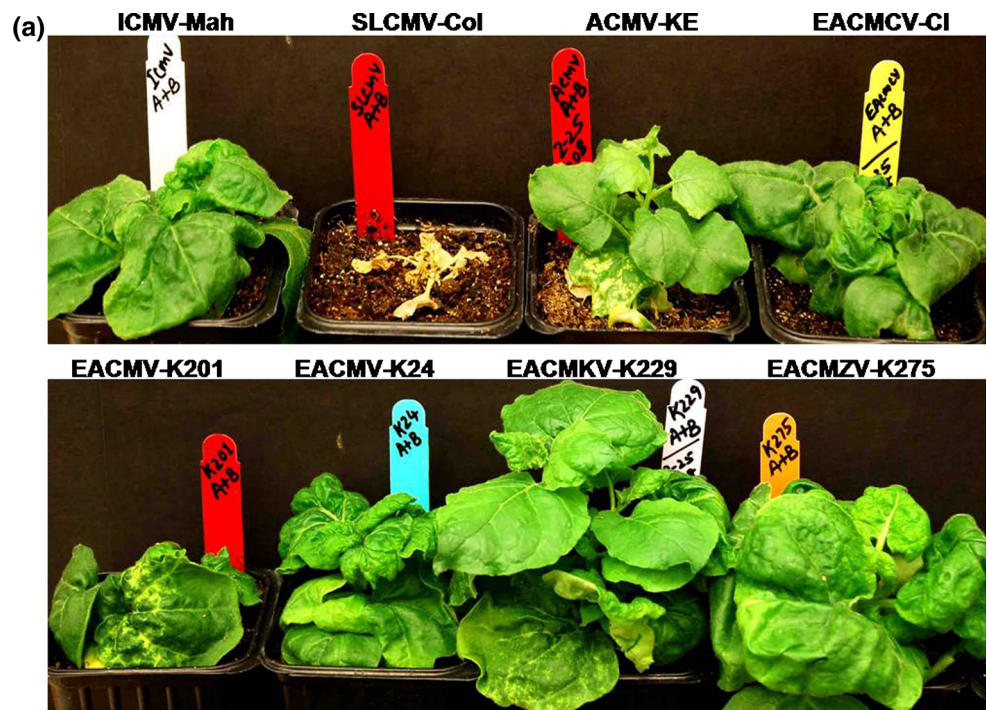
## Results

### CMGs produce contrasting symptom phenotypes and infectivity patterns

All the infectious clones of the CMGs were studied for their infection dynamics by both particle bombardment and agro-inoculation (Fig. 1a). Depending on the time period required for appearance of symptoms in *N. benthamiana*,



**Fig. 1 a** Diverse patterns of symptoms obtained after agro-inoculation of different species and isolates of cassava mosaic geminiviruses (CMGs) in *Nicotiana benthamiana*. Photographs were taken 3 weeks post-inoculation. **b** Graphical representation of symptom progression of different CMGs in *N. benthamiana*. Based upon their symptom progression over time, the CMGs are classified into different groups in Table 1

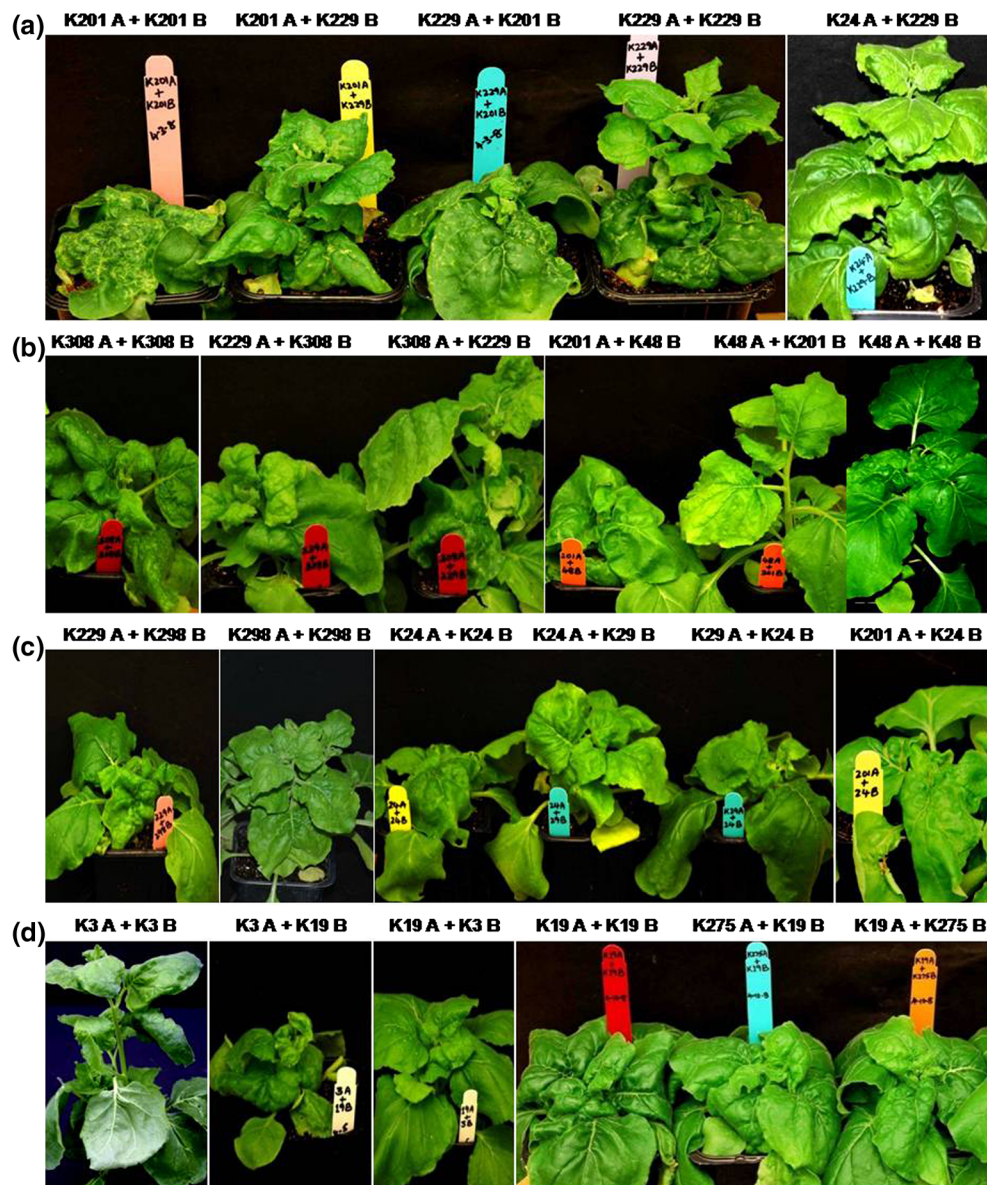


these CMGs are broadly classified into three groups: fast (clearly visible symptoms in 1–4 days post-inoculation), medium (4–8 dpi), and slow (>8 dpi) viruses (Table 1; Fig. 1b). In addition, these CMGs are also grouped into recovery and non-recovery types (Table 1). SLCMV-LK[LK:Col:98]-induced necrosis in *N. benthamiana* leading to its death and is thus grouped as a non-recovery virus, whereas cassava plants infected by it recover in due course of time [25].

We also studied the infectivity of CMGs in three different *Nicotiana* species (*N. benthamiana*, *N. tabacum*, and

*N. debneyi*). SLCMV-Col quickly infected and produced severe symptoms in both *N. tabacum* and *N. debneyi*, and ACMV-KE and ICMV-Mah produced mild symptoms, whereas none of the EACMV-like viruses including SACMV-ZA could infect *N. tabacum* and *N. debneyi*. Furthermore, we investigated the ability of EACMV-like viruses (EACMV-K201, EACMKV-K229, and EACMZV-K275) to infect the CMD-resistant cassava cultivar TME3, by particle bombardment (Supplementary Fig. 1). Unlike the localized infection spots produced by ACMV-CM on the inoculated leaves of TME3, the EACMV-like viruses





**Fig. 2** Change in symptom patterns exhibited by *Nicotiana benthamiana* after inoculation with cassava mosaic geminiviruses, when the DNA-B component was swapped **a** between the isolates of EACMV (K201 and K24) and EACMKV-K229, **b** among different isolates of EACMV (K24, K29, and K201), **c** among different isolates

of EACMKV (K229, K308, and K298), and **d** isolates of EACMZV (K3, K19, and K275). The symptomatic *N. benthamiana* plants were photographed 3 weeks post-inoculation. Refer Table 2 for summary of symptom phenotypes produced by different combinations of DNA-A and DNA-B of CMGs

gave systemic symptoms, with EACMV-K201 producing most severe symptoms, which eventually recovered in contrast to the non-recovery phenotype exhibited by EACMV-K201 in the CMD-susceptible cassava cultivar TMS 60444 (Supplementary Fig. 1).

DNA-B component of CMGs significantly contributes to the nature of symptoms

To know the contribution of each genomic component toward the nature of symptoms, we conducted pseudo-

recombination studies in *N. benthamiana* by swapping the cognate DNA-A and DNA-B components of CMGs. For this, we inoculated the DNA-A and DNA-B components of different isolates of CMGs in different combinations. The swapping of DNA-A and DNA-B components between different isolates of CMG species resulted in drastic change in the symptom phenotype (Table 2; Fig. 2).

The DNA-B component of EACMKV-K229 produced recovery phenotypes when it was co-inoculated with its cognate DNA-A (EACMKV-K229) and also with DNA-A from EACMV-K201, EACMV-K24, and EACMKV-K308

**Table 1** Classification of CMGs (cassava mosaic geminiviruses) into different groups based upon their symptom phenotypes and infection dynamics in *Nicotiana benthamiana* (Fig. 1b)

Time for visible symptom development	Fast (4 dpi)	Medium (4–8 dpi)	Slow (>8 dpi)
No recovery	SLCMV-LK[LK:Col:98]*	EACMV-KE[KE:Kat:K24:01]*, EACMV-KE[KE:Msa:K201:02]*, EACMV-KE[KE:Mis:K27:01], EACMCV-CI[CI:98]*, EACMZV-[KE:Kib:K275:02]*, EACMV-KE[KE:Boa:K48:01]#, EACMV-KE[KE:Mig:K268:02], EACMKV-[KE:Mat:K308:02]#, EACMKV-[KE:Mit:K298:02]#	EACMV-UG[KE:Wot:K282:02], EACMZV-[KE:Fel:K19:01]#
Recovery	ACMV-[CM:DO2:98], ACMV-[KE:844:82]*	EACMKV-[KE:Keh:K229:02]*, SACMV-ZA	ICMV-IN[IN:Mah:88]*, EACMV-KE[KE:Kib:K29:01], EACMV-KE[KE:Mig:K268:02]

The CMG isolates highlighted in gray were not subjected to molecular analysis but were only studied for their infectivity *dpi* days post-inoculation of the virus

\* These virus symptoms are shown in Fig. 1

# These virus symptoms are shown in Fig. 2

**Table 2** Symptom phenotypes produced by cassava mosaic geminiviruses in *Nicotiana benthamiana*, when the DNA-B component was swapped between (a) EACMV-K201 and EACMKV-K229, (b) different isolates of EACMKV (K229, K308, and K298) and (c) isolates of EACMZV (K19 and K275) (Fig. 2)

Nature of symptom (recovery/severity)	DNA-B									
	EACMV				EACMKV			EACMZV		
	K201	K48	K24	K29	K229	K308	K298	K3	K19	K275
<b>DNA-A</b> EACMV	<b>K201</b>	NR/s	NR/s	NR/s	MR/s			No trans-replication between EACMV and EACMZV		
	<b>K48</b>	mi	NR/s							
	<b>K24</b>			NR/s	NR/m	R/s				
	<b>K29</b>			NR/s						
EACMKV	<b>K229</b>	NR/s			R/s	NR/m	NR/s	No trans-replication between EACMKV and EACMZV		
	<b>K308</b>				R/m	MR/m				
	<b>K298</b>						NR/m			
EACMZV	<b>K3</b>	No trans-replication between EACMV and EACMZV			No trans-replication between EACMKV and EACMZV			NR/mi	NR/s	
	<b>K19</b>							NR/mi	NR/s	NR/m
	<b>K275</b>								NR/s	NR/m

Based on recovery phenotypes, the symptoms were classified as, *R* Recovery, *MR* moderate recovery, *NR* non-recovery, and based on symptom severity, the symptoms were classified as, *s* severe, *m* moderate, *mi* mild, and *n* no symptom

(Fig. 2). However, the DNA-B component of EACMKV-K308, another isolate of EACMKV, produced non-recovery phenotype when co-inoculated with DNA-A of EACMKV-K229 (Fig. 2). Similarly the DNA-A and DNA-B components of different isolates of the CMG species EACMV and EACMZV differentially manifest the symptom phenotypes.

The isolates of EACMV, namely K24, K29, K48, and K201, produced varying levels of symptom severities when their DNA components were co-inoculated in different combinations. Similarly in the case of isolates of EACMZV, the DNA-B of isolate K19 produced most severe symptoms followed by the DNA-B of K275 and K3 (Fig. 2).

DNA-A component of some of the CMGs displays reminiscent monopartite behavior on agro-inoculation in *N. benthamiana*

Earlier studies have shown that SLCMV exhibits characteristic features of a monopartite begomovirus, and it is hypothesized that the DNA-A component of SLCMV acquired ICMV DNA-B to become a bipartite begomovirus [11]. To know if in addition to SLCMV, other species of CMGs also acquired their cognate DNA-B component from other bipartite begomovirus species [10, 11], and if these CMGs still exhibit a reminiscent monopartite feature like SLCMV DNA-A, we studied the ability of DNA-A component alone to replicate and systemically spread in *N. benthamiana*. The DNA-A components alone of ICMV-Mah, ACMV-KE, SACMV-ZA, EACMV-K201, EACMKV-K229, EACMZV-K275, and EACMCV-CI were agro-infiltrated in *N. benthamiana*. Of these CMGs, DNA-A of ACMV-KE, EACMV-K201, EACMZV-K275, and ICMV-Mah accumulated at detectable levels in the systemic non-inoculated leaves, and DNA-A of SACMV-ZA accumulated in smaller amounts (Supplementary Fig. 2). Furthermore, the DNA-A component of EACMV-K24, EACMCV-CI, and EACMKV-K229 could not accumulate to detectable levels in the inoculated plants. Our studies with DNA-A component of SLCMV-Col did not produce any symptoms when inoculated by particle bombardment, but symptoms' characteristic of monopartite begomoviruses was obtained only by agro-inoculation as reported by Saunders et al. [11], thus indicating that virus inoculation through *Agrobacterium* was a key for symptom production [26]. Thus, the infection by DNA-A component alone of CMGs was possible only through agro-inoculation but not by particle bombardment or other methods of mechanical inoculation.

siRNAs of CMGs show a variable pattern of accumulation across the infected host plant

Analyses for accumulation of viral siRNAs, mRNA, and DNA across the symptomatic plants infected by different CMGs showed a gradient of accumulation of siRNAs (Fig. 3a, b). In all the cases, the oldest/lowest symptomatic leaf showed the highest accumulation of siRNA, and the youngest symptomatic leaves showed reduced accumulation of siRNA. However, there was no correlation between the recovery phenotype and siRNA levels or its distribution. In the case of ACMV-KE, EACMV-K201, and EACMKV-K229, there was gradual reduction in the amount of siRNA from the bottom to the top of symptomatic *N. benthamiana*. In contrast, in the case of EACMZV-K275, there was no significant change in the amount of siRNA across the infected plant. However, in the case of ACMV-KE showing complete recovery of

symptoms, the drop in the levels of siRNA was more drastic compared to gradual reduction in the levels of siRNA accumulation in EACMV-K201 producing non-recovery phenotype and EACMKV-K229 producing recovery phenotype. The amount of viral siRNA produced across the plants was fairly proportional to the extent of viral transcript and viral DNA accumulation in the case of ACMV-KE (Fig. 3a). However, in the case of EACMV-K201 and EACMKV-K229, which are non-recovery type viruses, there was an inverse correlation between the amount of siRNA and the levels of viral mRNA or viral DNA. To summarize, the recovery of infected plant was not correlated with continuous production of siRNA over the time assayed, except for EACMV-K275 (Fig. 3a). In some of the old symptomatic leaves of *N. benthamiana*, it was not possible to extract good quality of mRNA and DNA, resulting in poor quality of hybridizations (Fig. 3a).

Additionally, we checked for the polarity and strand origin of siRNAs across the genomes of ACMV-KE, a recovery type virus and EACMV-K201, a non-recovery type virus. The studies showed that the siRNAs were derived from both the polarities of viral genome and also from the ORFs present on both virion and complementary strands of the dsDNA of replicating CMGs (Fig. 3b). The siRNAs in all the cases showed a conserved trend of accumulation pattern across the infected plant. However, the siRNA populations derived from the DNA-A component were in larger amounts than the DNA-B component, and the siRNAs derived from the complementary strand were in larger amounts than those derived from the virion strand of CMGs.

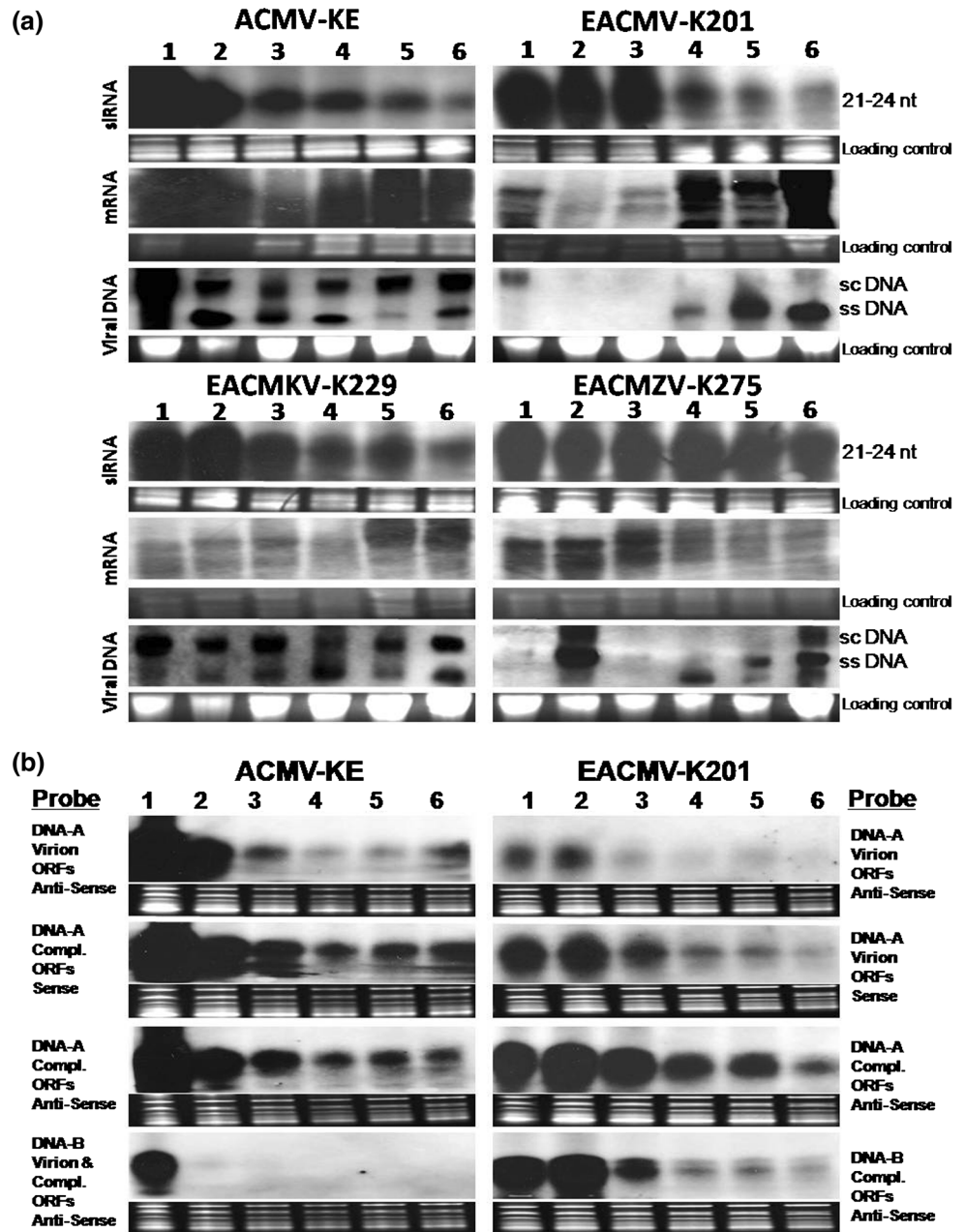
Our results show, irrespective of recovery type CMGs (ACMV-KE or EACMKV-K229) or non-recovery type CMG (EACMV-K201), and there was a gradual reduction in the levels of siRNA from bottom (older symptomatic leaves) to the top of the plant (younger symptomatic/recovered and non-symptomatic leaves), although such reduction in siRNA levels was not significant in the case of EACMZV-K275. Thus, it is difficult to make any correlation in the amount of siRNA produced across the plant and the recovery from the viral symptoms.

CMGs display diverse patterns of siRNA origin and density across their genomic components

The origin and density of the virus-derived siRNAs were mapped across both the genomic components of CMGs, DNA-A and DNA-B, by reverse-Northern hybridizations. The pattern of siRNA distribution was different for all the CMGs, indicating a high amount of diversity in the origin and accumulation of siRNAs across the genomes of different species and isolates of CMGs (Fig. 4). The amount of siRNA accumulating in specific region/s of the viral



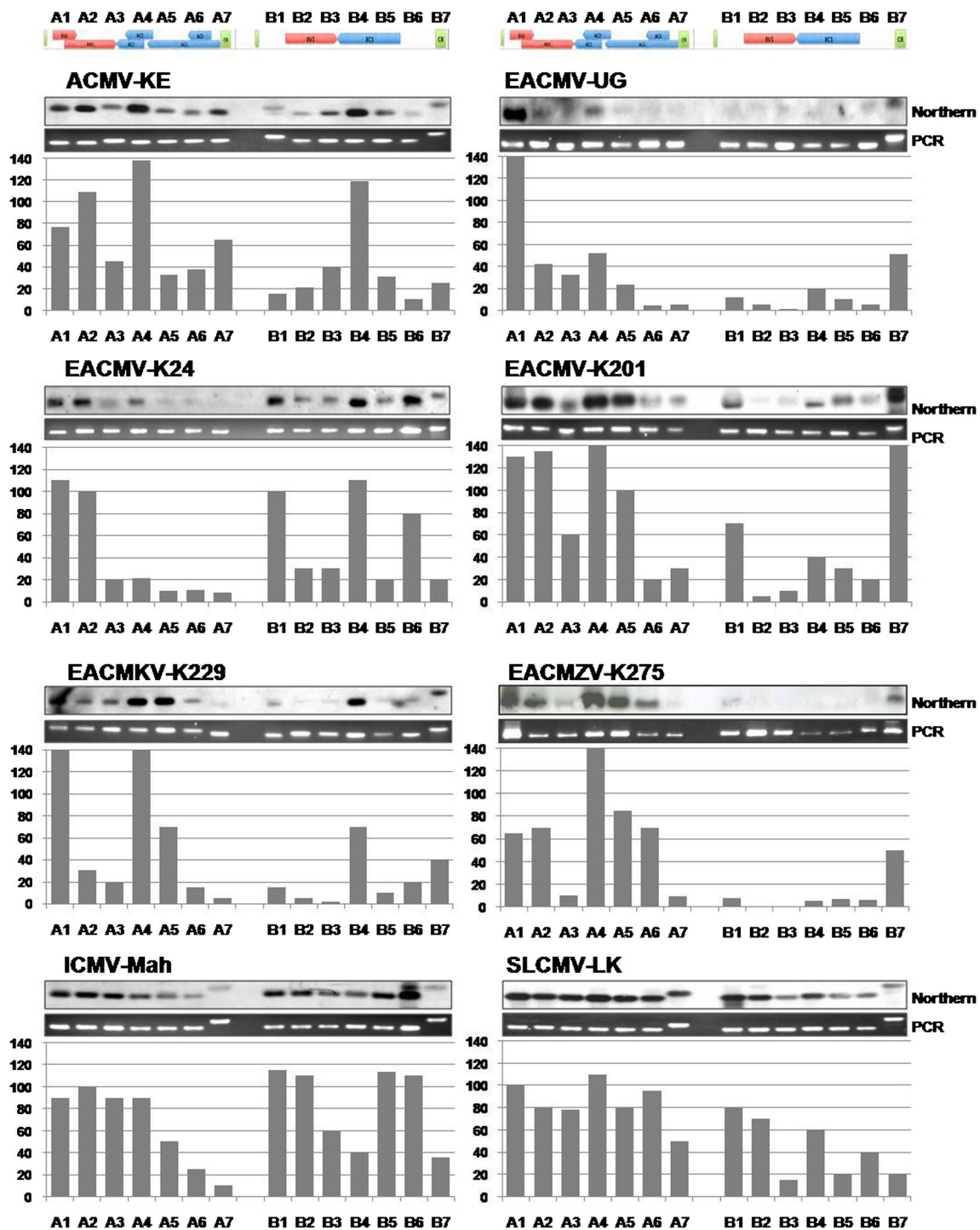
**Fig. 3 a** Accumulation of siRNA, mRNA, and viral DNA in individual leaves of *Nicotiana benthamiana*, from lower leaf (lane # 1) to the upper leaf of plant (lane # 6), infected with four different cassava mosaic geminiviruses exhibiting contrasting symptom phenotypes. **b** Polarity of siRNA gradients observed on hybridization with different probes homologous to virion sense, virion anti-sense, complementary sense, or anti-sense ORFs of DNA-A and DNA-B components of a recovery type (ACMV-KE) and non-recovery type (EACMV-K201) CMGs



genome is defined by the density of siRNAs, which is measured by the intensity of signals in reverse-northern hybridizations, and the signal strength is graphically represented beneath each blot (Fig. 4). In most cases, the DNA-A component had a higher density of siRNA compared to its DNA-B counterpart, but in the case of ICMV-Mah and EACMV-K24, the DNA-B component had a higher density of siRNAs in comparison to their cognate DNA-A component.

There was a significant variation in the densities of siRNAs across both DNA-A and DNA-B components of CMGs (Fig. 4). In most CMGs (ACMV-CM, ACMV-KE,

EACMV-K201, EACMKV-K229, and EACMZV-K275), the A4 fragment (1201–1600 nt) of DNA-A component showed the highest density followed by A2 (401–800 nt) and A5 (1600–2000 nt), whereas in the case of SLCMV-LK, the siRNAs mostly mapped uniformly across all the segments of DNA-A genome but not across the DNA-B component. Although in several instances, the fragments overlapping with the intergenic regions of both the DNA-A (A1 and A7) and DNA-B components (B1 and B7) showed significant amount of siRNAs, it was highly variable as evident from different replications of reverse-Northern hybridizations (data not shown). In the DNA-B component, the segments



**Fig. 4** The siRNA origin maps of cassava mosaic geminiviruses as obtained by reverse-Northern hybridization. PCR-amplified ~400 bp DNA segments from DNA-A component (A1–A7) and DNA-B component (B1–B7) of different CMGs were resolved in 1 % Agarose gel and were blotted and hybridized with 5' end-labeled

B4 (1201–1600 nt) and B2 (401–800 nt) corresponding to C-terminal of BC1 and N-terminal of BV1 showed high levels of siRNA in five and two CMGs, respectively, of the eight CMGs that were analyzed (Fig. 4).

siRNAs purified from CMG-infected *N. benthamiana*. Intensity of the signals as scored (0–140) using ImageQuant software is represented on the Y-axis, and the PCR-amplified DNA segments are indicated on the Y-axis

Different isolates of the same CMG species, i.e., the ACMV isolates: -CM and -KE and the EACMV strains/ isolates, -K201, -K24, and -UG showed different patterns of siRNA distribution across their genomes. However, both

the ACMV isolates (-CM and -KE) had high density of siRNAs in the A4 (AC2) and A2 (part of AV1) regions of the DNA-A component and B4 region of the DNA-B component (data not shown for ACMV-CM). Similarly in the case of EACMV isolates, A2 and A4 segments produced strong signals for siRNA accumulation, whereas in DNA-B, the B4 segment had the highest signal intensity. The two EACMV-like viruses EACMKV-K229 and EACMZV-K275 had the highest density of siRNA on the segment A4; however, there was significant variation in the distribution of siRNA across their genomes, in different replications. Although each CMG showed diverse and unique pattern of siRNA distribution across their genomes, it was difficult to make a correlation between the siRNA origin and distribution pattern and the nature of symptom phenotype exhibited by each CMG species/isolate. But overall, the segment A4 corresponding to AC2 in the DNA-A component and segment B-4 corresponding to part of BC1 in DNA-B component of the CMGs showed high levels of siRNA accumulation. There was no cross-hybridization of small RNA (15–30 nt) from mock inoculated *N. benthamiana* with the PCR segments of CMGs (data not shown).

## Discussion

CMD has become a major concern for cultivation of cassava in the African continent and the Indian sub-continent, and the increasing diversity of CMGs, through recombination and pseudo-recombination, has made it more challenging to control this menace [2, 5, 27]. Thus, it is important to study the vast diversity of CMGs, the molecular basis of this diversity, and also their epidemic implications. It is also important to understand the biological behavior of CMGs and to design an effective management strategy. Thus, for the first time, we have made a systematic effort to study the infection dynamics of several CMG species, their strains/isolates, using two different methods of virus inoculation, particle bombardment, and agro-inoculation.

Past studies have shown that agro-inoculation of DNA-A alone of ACMV-KE and Abutilon mosaic virus (AbMV), resulted in systemic movement and encapsidation of the viral genome, although no visible symptoms were produced [12, 28]. Saunders et al. [11] demonstrated that the inoculation of DNA-A component alone of SLCMV in *N. benthamiana* showed upward leaf-rolling and vein-thickening symptoms characteristic of a monopartite begomovirus [29]. These results led to the interesting hypothesis that SLCMV could represent an evolutionary link between the monopartite and bipartite begomoviruses [11]. Our experiments demonstrated that in addition to DNA-A of

ACMV-KE, the DNA-A of EACMV-K201 and EACMZV-K275 also showed significant accumulation, when inoculated alone. The DNA-A component of ICMV-Mah and SACMV-ZA also accumulated to detectable levels when it was agro-inoculated in *N. benthamiana*. However, of all the DNA-As of CMGs, the DNA-A of EACMZV-K275 alone produced mild symptoms due to its higher accumulation in comparison to others. So, these results may indicate that some of these CMGs might have had a monopartite origin with features reminiscent of monopartite begomoviruses and may have eventually evolved to become bipartite begomoviruses by acquisition of a DNA-B component [10, 30]. This is further supported by the fact that, in nature, some of the DNA-B components have been exchanged or a better fit has been obtained between the two genomic components originally belonging to two different species [10, 17, 31].

Previously, it has been reported that the BC1 (movement protein) of DNA-B component is a pathogenicity factor [32]. Studies by Bull et al. [17] showed that, depending on the nature of DNA-B component associated with the DNA-A component of EACMV-like viruses, a range of symptom phenotypes was obtained in natural infections of cassava. The pseudo-recombination studies undertaken by Bull et al. [17] were at the species level, and here, we report similar results, but for different isolates of three distinct CMG species, namely EACMV, EACMKV, and EACMZV. Our results clearly show that the DNA-B component significantly contributes to the symptom severity and the symptom phenotype (recovery or non-recovery phenotypes). These observations are consistent with the past reports available for AbMV, Mungbean yellow mosaic virus (MYMV), Tomato leaf curl New Delhi virus (TLCNDV), and Tomato leaf curl Gujarat virus (ToLCGV), in which case the nature of DNA-B associated with the DNA-A component had a profound influence on the symptom severity [31, 33, 34].

Past studies have also shown that the defective DNA-B ameliorated the symptoms of CMG-infected *N. benthamiana*, thus establishing the dependence of symptom phenotype on the movement of virus within the plant [35, 36]. The sequence analyses of geminiviruses across the globe demonstrate that there is a greater variation among the sequences of DNA-B components, when compared to the sequences of DNA-A components, and that exchange of genomic component is more widespread than previously demonstrated and is confined to the begomoviruses from the Old World [10].

Plant viruses can be conveniently described based upon their host range which helps in better understanding of their etiology. Host range studies showed that of all the CMGs analyzed, none of the EACMV-like viruses (EACMV, EACMKV and EACMZV) could infect *N. tabacum* or *N.*



*debneyi*, indicating that the cassava infecting EACMV-like viruses may have different evolutionary histories. It was also interesting to see that SACMV, which did not infect other *Nicotiana* species, like the EACMVs, clustered with EACMV-like viruses, rather than ACMV, when subjected to phylogenetic analysis [6]. Studies have shown that most geminiviruses could replicate in protoplasts derived from different plant species, but they fail to induce symptoms when agro-inoculated into the non-host plant species, indicating that the host range restriction is not due to an absolute inhibition of viral replication but could be determined by their ability to move to adjacent cells and tissues [37, 38].

Thus, the above studies on characterization of CMGs clearly demonstrate that each CMG species/isolate is unique in its behavior. Through series of experiments we try to understand the fundamental behavior of different CMGs in different host plants and also the factors contributing to their virulence and the nature of symptom phenotype. One of the finding from this study demonstrated that EACMV-K201 could infect the CMD-resistant cassava genotype TME3 [39], although TME3 eventually recovered from the infection in contrast to the absence of symptom recovery in the CMD-susceptible cassava cultivar TMS60444. The TME3 genotype is known to have the CMD-resistant locus CMD-2 which may help it to recover from EACMV infection or resist the ACMV infection [39]. Such information on infectivity of CMGs may help in developing improved strategies for their management through breeding or genetic engineering.

Gene silencing triggered by the virus-derived siRNAs helps the virus-infected plants to recover from the infection and eventually produce recovery phenotypes in the new growth [2, 14, 16]. In response to the infection by plant viruses, plants use the Dicer-like (DCL) proteins as the key enzymes of PTGS to regulate expression of viral transcripts and thus mediate the defense against viruses [40]. Different classes of DCLs process the dsRNA formed through the activity of RNA-dependent RNA-polymerases (RdRp) to produce varying sizes of siRNAs [41, 42]. The population of siRNAs obtained after viral infection depends upon the nature of the virus and its location within the host cell (nucleus or cytoplasm) and the nature of DCLs processing it [40]. The analysis of siRNA accumulation across the *N. benthamiana* plants infected with different CMGs showed different gradients of siRNA accumulation. In most cases, there was reduction in the levels of siRNAs from the bottom to the top of plant. In case of ACMV-KE, the reduction in the level of siRNA was sudden, whereas it was gradual in the cases of EACMV-K201 and EACMKV-K299, while EACMZV-K275 showed fairly uniform levels of siRNAs across the entire plant. These results do not correlate with the recovery patterns demonstrated by the CMGs in *N.*

*benthamiana* (Fig. 1). In addition to PTGS, the epigenetic modification of the viral genome may also contribute for the recovery of the virus-infected plant [43, 44]. Ghoshal and Sanfacon [45] showed that recovery of a Tomato ringspot virus (nepovirus)-infected plant was due to reduction in steady-state levels of coat proteins and the movement proteins because of Ago1-dependent repression of translation in viral RNA-2. Our studies also showed that the siRNAs were derived from both the polarities of the replicating viral DNA clearly indicating the formation of dsRNA. Although geminiviruses do not have any dsRNA phase in their life cycle, past studies strongly suggest for formation of dsRNA either from viral DNA or the ssRNA through RDR6 activity [46]. It has also been shown that the plants deficient in RDR6 activity hyperaccumulate viruses [47, 48].

Furthermore, we characterized the siRNAs generated from these diverse CMGs, by mapping their origin and distribution across the viral genomes and also determined the polarity of siRNAs [16]. Our studies showed that all the CMGs had a unique pattern of siRNA origin and distribution across the DNA-A and DNA-B components. The different patterns of siRNA origin maps obtained for different CMGs could be because of differences in their genome sequences, particularly the GC content, the secondary structure of transcripts encoded by them, or the amount of transcript accumulated due to their variable expression levels [49–52]. In most CMGs (five of eight), the AC2 and the C-terminal of AC1 had the highest density of siRNAs, whereas in the case of DNA-B component of some CMGs, the region corresponding to C-terminal of BC1 had higher density of siRNA. Coincidentally both AC2 and BC1 are the viral genes located on the complementary strand of DNA-A and DNA-B components, respectively, which are transcribed by early and strong promoters, thus, producing higher levels of transcripts and resulting in enhanced siRNA accumulation [51]. The pattern of origin and distribution of siRNAs across the genomes of CMGs was fairly reproducible in different replications of the experiment, except for the intergenic region which was highly variable in its density of siRNAs across replications (data not shown). This variation could be due to differential methylation patterns of the geminivirus intergenic region during different phases of virus life cycle [44]. Studies by Raja et al. [43] have shown that the methylation of intergenic regions of geminiviruses is an important mode of host defense against the viruses. Deep sequencing of the siRNA libraries constructed from the CMG-infected plants may further enhance the understanding of the distribution patterns of different size classes of siRNAs [53, 54]. Thus, the comparison of siRNA distribution patterns obtained by reverse-Northern hybridizations and deep sequencing, and identification of differences using both the methods might show considerable differences. For

example, methylated siRNAs which are not amenable for cloning may show up in the Northern hybridizations, but not in deep sequencing technologies [55]. Thus, this study shows that each virus species and their isolates are unique with unique maps of siRNA origin and distribution, which is determined by the sequence composition of the viral genome and the expression level of each gene. This in turn may be determined by the rate of virus replication and accumulation of viral nucleic acids. In this study, it was not possible to make any correlation between the siRNA origin/distribution patterns and the pattern of symptoms exhibited by CMGs. However, the information obtained in the above studies will help in identifying the appropriate RNAi target sequences of CMGs for an effective RNAi-based transgenic management.

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