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PCR-RFLP analysis indicates that recombination might be a common occurrence among the cassava infecting begomoviruses in India

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Abstract Cassava mosaic disease (CMD) is caused in India by two bipartite begomoviruses, *Indian cassava mosaic virus* (ICMV), and *Sri Lankan cassava mosaic virus* (SLCMV). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used as a rapid means of investigating the molecular diversity of ICMV and SLCMV in 38 samples of CMD-affected cassava plants under field conditions in new areas of cassava cultivation, along with traditional areas in southern India. A very large proportion of the samples showed SLCMV, based on a discriminatory PCR between SLCMV and ICMV, reported earlier. PCR-RFLP analysis of three regions of viral DNA indicated that in most samples, although the *AC1* and the *AV1* resembled SLCMV, as expected, the intergenic regions (binding site for host replication machinery) resembled ICMV more closely, indicating recombination events between ICMV and SLCMV. Results also indicate that the *AC1* is more conserved within SLCMV compared to the *AV1* gene.

Keywords ICMV · SLCMV · PCR-RFLP · Recombination

Introduction

Cassava mosaic disease (CMD) is widespread in Africa and southern India, characterized by mosaic and deformation of the leaves. A begomovirus (family: *Geminiviridae*), was first shown to be associated with CMD in 1983 [1]. The genus begomovirus consists of large number of members; all transmitted by whitefly (*Bemisia tabaci* Genn.) to dicotyledonous hosts [2]. Based on their organization, they could be either mono- (containing only one DNA molecule called DNA-A) or bipartite (containing two DNA molecules: DNA-A and DNA-B). DNA-A codes for the coat protein (CP/AV1) and a protein of uncertain function (AV2) in the viral-sense strand and replication associated protein (Rep/AC1), transcriptional activator (TrAP/AC2), replication enhancer (Ren/AC3) and a possible silencing suppressor (AC4) in the complementary-sense strand. DNA-B codes for nuclear shuttle protein (NSP/BV1) and movement protein (MP/BC1) in the viral-sense and complementary-sense strands, respectively.

In India, two begomoviruses, *Indian cassava mosaic virus* (ICMV, [5]) and *Sri Lankan cassava mosaic virus* (SLCMV, [6]) are associated with CMD, according to surveys conducted in southern India, mainly in the states of Kerala and Tamil Nadu in the southwestern coast and in the south of the country. Of the two, SLCMV was found to be more prevalent in the cassava growing areas, while ICMV was found to be isolated in specific pockets [4]. Between DNA-A of ICMV and SLCMV, the most diverse region is the intergenic region (IR), (which does not contain any coding region, but contains the elements controlling replication and transcription) and the most conserved region is the *CP*. As such, SLCMV is believed to be a product of evolution, in which, from being a potential monopartite *Begomovirus*, it has acquired the DNA-B of ICMV [3]. Both ICMV and SLCMV are known to contain molecular variants [4].

Here, PCR-RFLP analysis of 38 CMD-infected cassava samples, collected over a period of 5 years (2002–2007)

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from cassava growing regions of southeastern, southwestern and southern India (Andhra Pradesh, Kerala and Tamil Nadu), was carried out, focusing on three different regions of the viral genome: IR, *AC1* gene and *AV1* gene, using three different enzymes. We conclude that (i) most samples indicated the presence of viruses which were recombination products of ICMV and SLCMV, (ii) IR region of ICMV and, *AC1* and *AV1* region of SLCMV is quite diverse and (iii) there exists a possible recombination hotspot between the IR and *AC1* region of the genome of ICMV/SLCMV.

Materials and methods

Source of virus-infected material

Field visits were made to cassava plantations and stem-cuttings were collected from apparently CMD-infected (depending on visual symptoms of leaf curling, crinkling and mosaic) cassava plants. These cuttings were maintained in soil inside an insect-proof greenhouse under a 16/8 h photoperiod, 30 ± 2 °C temperature and 70–80 % humidity.

Isolation of total nucleic acids, polymerase chain reaction and restriction digestion

Total nucleic acids were isolated from leaves sprouted from the cuttings and multiplex PCR was performed for differential detection of ICMV and SLCMV according to Patil et al. [4]. For PCR-amplification of IR, *AC1*, and *AV1* regions (Fig. 1), primers were designed based on nucleotide sequence of ICMV and SLCMV DNA-A (Table 1). PCR amplifications were performed in a volume of 20 µl using Taq-DNA polymerase (New England Biolabs, USA) in a TC-512 thermocycler (Techne, UK) according to the manufacturer's instruction. dNTPs were procured from Roche diagnostics (USA) and primers were commercially synthesized (Sigma-Aldrich, India). Thirty cycles of amplifications were performed with denaturation at 94 °C for 30 s, annealing at respective temperature for 30 s (Table 1), extension at 72 °C for 1 min; excluding initial denaturation for 5 min at 94 °C and final extension for 10 min at 72 °C. As positive control for ICMV and SLCMV, full length clones AY730035 and AJ579307 for ICMV and SLCMV, respectively were used. As a negative control, leaves from asymptomatic plant, maintained in insect-proof condition were used. Three four-base cutter restriction enzymes: *Hpa*II (Fermentas, Lithuania), *Rsa*I (NEB), and *Taq*I (Fermentas) were used for digesting the DNA completely according to instructions of the manufacturers.

Electrophoresis

The PCR products for the detection of ICMV and SLCMV were resolved by electrophoresis according to [4]. For PCR-RFLP, a 7 % polyacrylamide gel in a vertical gel apparatus (Owl, USA), was used for electrophoresis for 5–6 h at 30 mÅ constant current. As size marker, ~100 ng of 50 bp DNA ladder (NEB) was used. After the electrophoresis was over, the gel was stained with ethidium bromide using standard procedure [7] and photographed in a UV–Vis gel documentation system (Quantity-One software, BioRad, USA).

Data-analysis

RFLP data were analysed using the PAST package in its excel-interface, scoring the bands "1" and "0" for the presence and absence, respectively, and similarity dendrograms were generated using the Jaccard's similarity matrix in the same package in paired-group method. Visual modifications were done in the generated dendrograms in Microsoft Power-point for better clarity without altering the original results.

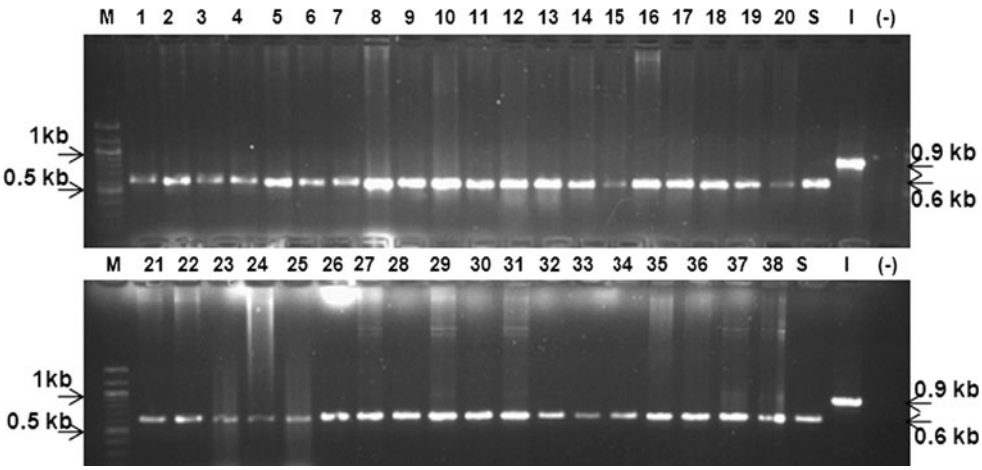

Fig. 1 PCR amplification to detect the begomoviruses from naturally infected cassava leaf samples. *M* size marker, 100 bp ladder, amplification using cloned DNA of ICMV and SLCMV are indicated as *I* and *S* respectively, (−), PCR using genomic DNA from asymptomatic plant

Table 1 ICMV-like (I) or SLCMV-like (S) PCR-RFLP patterns in the three regions of resident begomoviruses of mosaic-affected cassava leaves collected from three states of India

Sample no.	Collected from (states)	AC1	IR	AV1
1	Kerala	S	I	S
2	Kerala	S	I	S
3	Kerala	S	S	S
4	Kerala	S	I	S
5	Tamil Nadu	S	S	S
6	Tamil Nadu	S	S	S
7	Tamil Nadu	S	I	S
8	Tamil Nadu	S	I	S
9	Andhra Pradesh	S	S	S
10	Andhra Pradesh	S	S	S
11	Andhra Pradesh	S	S	S
12	Andhra Pradesh	S	S	S
13	Andhra Pradesh	S	I	S
14	Andhra Pradesh	S	I	S
15	Andhra Pradesh	S	S	S
16	Andhra Pradesh	S	S	S
17	Andhra Pradesh	S	I	S
18	Andhra Pradesh	S	I	S
19	Andhra Pradesh	S	I	S
20	Andhra Pradesh	S	I	S
21	Andhra Pradesh	S	I	S
22	Andhra Pradesh	S	I	S
23[a]	Andhra Pradesh	S	I	I
24	Andhra Pradesh	S	I	S
25	Andhra Pradesh	S	I	S
26	Andhra Pradesh	S	I	S
27	Andhra Pradesh	S	I	S
28	Andhra Pradesh	S	I	S
29	Andhra Pradesh	S	I	S
30	Andhra Pradesh	S	S	S
31	Andhra Pradesh	S	I	S
32	Andhra Pradesh	S	I	S
33	Andhra Pradesh	S	S	S
34	Andhra Pradesh	S	I	S
35	Andhra Pradesh	S	I	S
36	Andhra Pradesh	S	I	S
37	Andhra Pradesh	S	S	S
38	Andhra Pradesh	S	I	S

[a] Unique PCR-RFLP pattern

Results

Detection of ICMV versus SLCMV

Multiplex PCR for differential detection of ICMV from SLCMV was carried out to differentiate between ICMV and SLCMV, using the total DNA isolated from 38 different cassava samples as templates. All the 38 samples gave an amplicon of ~600 bp, representing the region flanked by 5′ end of AC1 and 5′ end of AV1, indicating the presence of only SLCMV in the samples, as reported earlier [4]. No sample showed a 900 bp amplicon, indicative of ICMV. This clearly shows that all the samples used for the analysis are infected by SLCMV alone (Fig. 1).

Detection of recombination between ICMV and SLCMV

The overall RFLP patterns (a representative shown in Fig. 2) for all the three PCR-amplified regions are summarized in Table 1 and Fig. 3. From this Table, it is clear that in case of samples #3, 5, 6, 9, 10, 11, 12, 15, 16, 30, 33, and 37 (12 out of 38), all the regions amplified gave an RFLP-pattern similar to that of SLCMV; but for the rest 26 samples, the three viral DNA regions showed patterns similar to both ICMV and SLCMV; 25 (#1, 2, 4, 7, 8, 13, 14, 17, 18, 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 31, 32, 34, 35, 36, and 38) contained IR region similar to ICMV, while the *AC1* and *AV1*regions were similar to SLCMV. One sample (#23, shown in bold in Table 1) contained the IR and *AV1* region resembling ICMV, while the *AC1* region resembled SLCMV. The data indicate that the first 12 samples (#3, 5, 6, 9, 10, 11, 12, 15, 16, 30, 33, and 37) are SLCMV and the other samples are recombinants of ICMV and SLCMV. It is also clear in case of all the 26 recombinants that while two of the genomic regions analysed [*AC1* and *AV1*; except in one case (sample no #23)] are similar to SLCMV, the IR is similar to ICMV. This indicates a recombination of the IR of ICMV in SLCMV background. The case of sample no #23 is a special one which might be a case of recombination of SLCMV *AC1* in ICMV background. The fact that in none of the cases, *AC1* resembling SLCMV was found in case of the recombinants, the *AC1* gene of SLCMV might be preferred over the same of ICMV during evolution. Therefore, it is also evident that there is likely to be a recombination hotspot between the *AC1* gene and the IR.

Detection of micro-variants of ICMV and SLCMV

In detailed analysis of the dendrograms generated using the restriction pattern of all the three enzymes (*Hpa*II, *Rsa*I, and *Taq*I) for the IR, *AV1*, and *AC1* separately (Fig. 4a–c), it was seen that the restriction pattern of IR from 26 samples were closer to the restriction pattern of IR of ICMV with more than 60 % similarity while the pattern in 12 samples was similar to the same of SLCMV. Out of the former 26 samples, 17 samples (#14, 4, 1, 8, 13, 17, 18, 21, 25, 26, 27, 28, 31, 32, 34, 35, and 36) showed 100 % similarity to ICMV. The rest of the samples shared different amounts of similarity to the

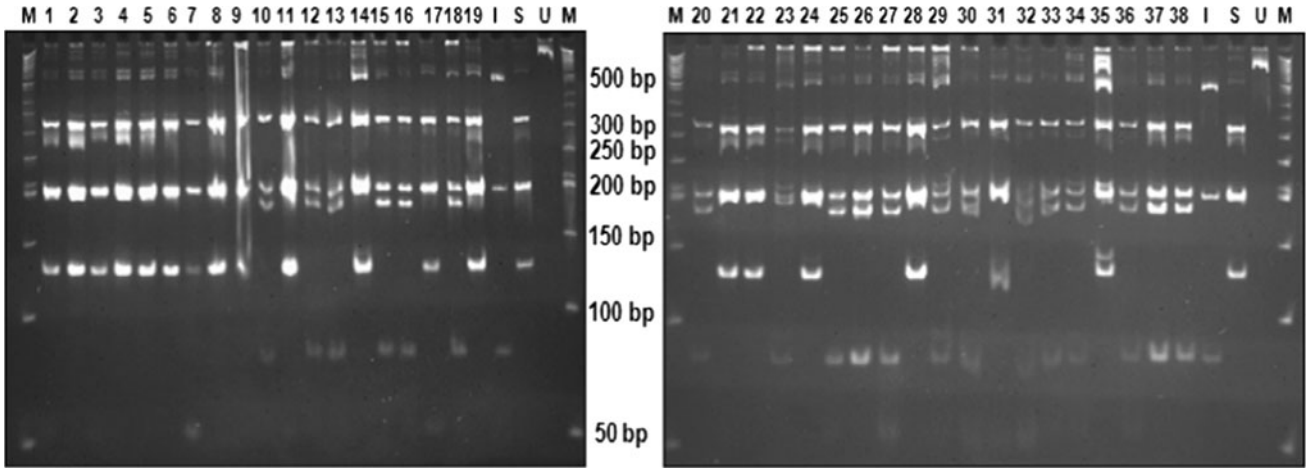

Fig. 2 PCR-RFLP patterns of ICMV-type and SLCMV-type viral DNAs from naturally infected cassava samples (for *AV1*/*Rsa*I). *M* size marker, 50 bp ladder, *I* cloned ICMV DNA control, *S* cloned SLCMV DNA control, *U* undigested PCR product. The sizes of the DNA markers are indicated in *base pairs*

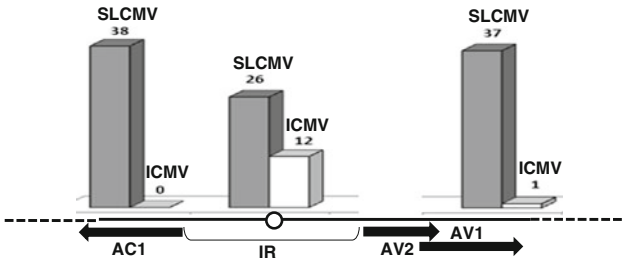

Fig. 3 Graphical representation of number of samples showing SLCMV-/ICMV-type PCR-RFLP patterns shown along the map of the viral genome. The numbers on the top of the bars indicate the number of samples showing a particular type of pattern. The ORFs and IR are shown as solid arrows; and the "ori" site is depicted as an open circle

known ICMV pattern, which ranged from 75 % (#23) to ~64 % (#7, 22, 2 and 20), revealing the presence of multiple micro-variants within the ICMV-like group. Similarly, among the SLCMV-like group consisted of 12 samples (#10, 3, 5, 9, 11, 12, 15, 16, 30, 33, 37, and 6), while 11 samples showed 100 % identity of patterns to SLCMV, one sample (#6) was a micro-variant of SLCMV, showing only ~77 % similarity to the same (Fig. 4).

Considering the restriction pattern of the *AV1* amplicon, 37 samples showed at least 40 % similarity to that of SLCMV while only one sample (#23) showed ~70 % similarity to ICMV. Among the 37 samples showing similarity to SLCMV, none showed 100 % identity to SLCMV; however, five samples (#21, 4, 5, 8, 19; which are 100 % similar to each other) showed ~90 % similarity to SLCMV. Although multiple samples among the rest of them were identical to each other, as in case of the IR, micro-variants were found to be present among them sharing similarity ranging from a maximum of ~65 % to a minimum of ~41 %.

The restriction pattern of the *AC1* was similar to the pattern of IR of ICMV showing no significant similarity to any of the samples, while 28 samples (#3, 1, 7, 8, 9, 10, 11, 12, 13, 14, 15, 19, 20, 21, 22, 23, 24, 26, 27, 28, 29, 31, 32, 33, 34, 35, 36, and 37) showed 100 % similarity to that of SLCMV. As in case of IR and *AV1*, micro-variants, however were present in this case also. For example, while two variants (#2, 36) showed a maximum of ~90 % similarity to SLCMV, three samples (#4, 5, 6) showed only ~45 % similarity to SLCMV, the rest showing intermediate values of similarity.

Discussion

It was earlier reported, that although both ICMV and SLCMV were associated with CMD in India, SLCMV is more widespread than ICMV [4, 8]. SLCMV is speculated to have had a previous non-cassava host from which it might have jumped to cassava and "captured" ICMV DNA-B [3]. The speculation is based on the observation that SLCMV DNA-A, on its own, is able to produce symptoms in experimental plants such as *Nicotiana benthamiana*, indicating a potential monopartite nature. Considering these facts, and the observation here and elsewhere that SLCMV and SLCMV-like isolates predominate in southern India, SLCMV seems to be more successful as a pathogen as compared to ICMV. The reported properties of SLCMV, such as increased aggressiveness, capability of trans-replicating DNA components of other viruses and infecting new hosts with satellite molecules [3] might have important contribution in this regard.

Based on the discriminatory PCR standardized earlier [4] to distinguish between ICMV and SLCMV, it is clear that most of the samples contained SLCMV, not ICMV. However, the PCR-RFLP patterns indicated that IR region had more resemblance to ICMV in the same samples. PCR-RFLP analysis, which covers the region analyzed

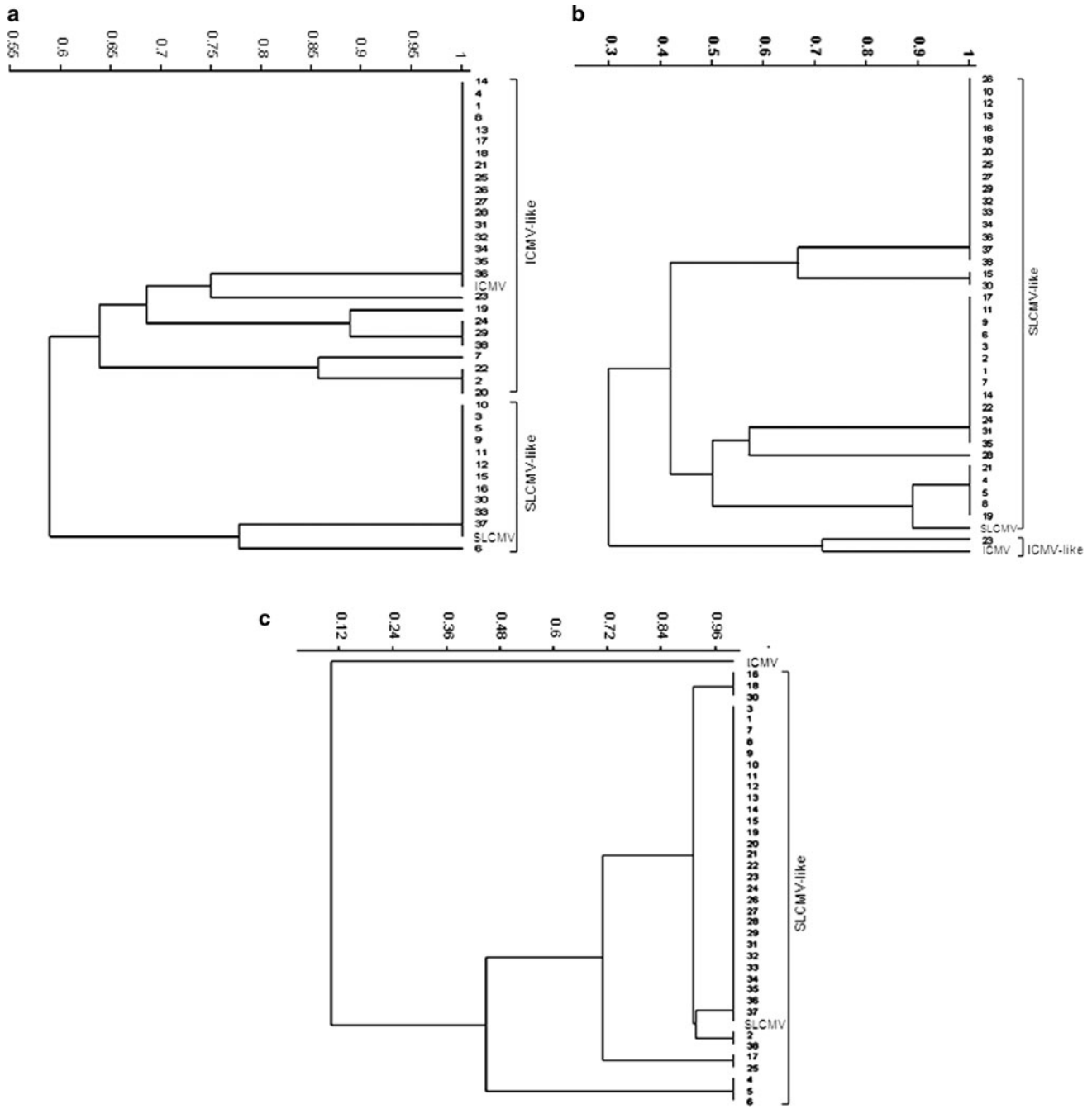

Fig. 4 Dendograms generated from the PCR-RFLP pattern. The known ICMV and SLCMV positive controls are marked in each, **a** IR, **b** *AV1*, and **c** *AC1*

uniformly and reflects the inherent DNA sequence heterogeneity, has been used to determine micro-heterogeneity in natural populations of viral quasi-species earlier [9]. Hence, it can be concluded that these patterns indicate strongly that the viral DNAs are a product of recombination between ICMV and SLCMV.

Recombination is a major factor, along with mutation and re-assortment, in contributing to the broadening of host-spectrum of viruses [10, 11]. Within geminiviruses, examples of the above phenomenon include the recombinant cassava geminivirus EACMV-Uganada, [12], *Maize streak virus* strain MSV-A, a recombination product between MSV-B and MSV-G/F [13], Tomato yellow leaf curl virus isolates [14], and *Cotton leaf curl Burewala virus* [15]. The mechanistic process underlying rolling circle replication of geminiviral DNA is believed to be the prime determinant of recombination [16], which was also implicated in the observation of recombinant defective DNA

molecules between ICMV and SLCMV in *N. benthamiana* [17]. Such processes may be responsible for the large number of recombinant geminiviruses in the recent past [18–24].

The present report is the first detailed investigation on recombination among cassava geminiviruses in southern India, using a large sample size, and extends a similar studies conducted earlier [4, 8]. The samples used in this study were collected from State of Andhra Pradesh in the southeastern coastal area of India and represents new areas being brought under cassava cultivation, a region not surveyed extensively for CMD. The widespread occurrence of SLCMV and its recombinogenic tendency pose grave threats to the cassava crop in southern India, especially in the newly cultivated regions, which highlights the urgent need to take effective antiviral measures for this crop.

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References

1. J. Stanley, M.R. Gay, Nucleotide sequence of *Cassava latent virus* DNA. Nature **301**, 260–262 (1983)
2. J. Stanley, D.M. Bisaro, R.W. Briddon, J.K. Brown, C.M. Fauquet, B.D. Harrison, E.P. Rybicki, D.C. Stenger. Geminiviridae, in *Virus Taxonomy. VIIIth. Report on the International Committee on the Taxonomy of Viruses*, ed. by L.A. Ball (Elsevier/Academic Press, London, 2005), pp. 301–326
3. K. Saunders, S. Nazeera, V.R. Mali, V.G. Malathi, R. Briddon, P.G. Markham, J. Stanley, Characterisation of *Sri Lankan cassava mosaic virus* and *Indian cassava mosaic virus*: evidence for acquisition of a DNA B component by a monopartite *Begomovirus*. Virology **293**, 63–74 (2002)
4. B.L. Patil, S. Rajasubramaniam, C. Bagchi, I. Dasgupta, Both *Indian cassava mosaic virus* and *Sri Lankan cassava mosaic virus* are found in India and exhibit high variability as assessed by PCR-RFLP. Arch. Virol. **150**, 389–397 (2005)
5. Y.G. Hong, D.J. Robinson, B.D. Harrison, Nucleotide sequence evidence for occurrence of three distinct whitefly-transmitted geminiviruses in cassava. J. Gen. Virol. **74**, 2437–2443 (1993)
6. N. Dutt, R.W. Briddon, I. Dasgupta, Identification of a second *Begomovirus*, *Sri Lankan cassava mosaic virus*, causing cassava mosaic disease in India. Arch. Virol. **150**, 2101–2108 (2005)
7. J. Sambrook, D.W. Russell, Molecular *Cloning: A Laboratory Manual*, 3rd edn. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001)
8. D. Rothenstein, R.W. Briddon, D. Haible, J. Stanley, T. Frischmuth, H. Jeske, Biolistic infection of cassava using cloned components of *Indian cassava mosaic virus*. Arch. Virol. **150**, 1669–1675 (2005)
9. R. Joshi, V. Kumar, I. Dasgupta, Detection of molecular variability in rice tungro bacilliform viruses from India, using polymerase chain reaction-restriction fragment length polymorphism. J. Virol. Methods **109**, 89–93 (2003)
10. S. Garcia-Andres, G.P. Accotto, J. Navas-Castillo, E. Moriones, Founder effect, plant host, and recombination shape the emergent population of begomoviruses that cause the tomato yellow leaf curl disease in the Mediterranean basin. Virology **359**, 302–312 (2007)
11. P. Lefeuvre, D.P. Martin, M. Hoareau, F. Naze, H. Delatte, M. Thierry, A. Varsani, N. Becker, B. Reynaud, J.-M. Lett, *Begomovirus* 'melting pot' in the south-west Indian Ocean islands: molecular diversity and evolution through recombination. J. Gen. Virol. **88**, 3458–3468 (2007)
12. X. Zhou, Y. Liu, L. Calvert, C. Munoz, G.W. Otim-Nape, D.J. Robinson, B.D. Harrison, Evidence thatDNA-Aof a *Geminivirus* associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J. Gen. Virol. **78**, 2101–2111 (1999)
13. A. Varsani, D.N. Shepherd, A.L. Monjane, B.E. Owor, J.B. Erdmann, E.P. Rybicki, M. Peterschmitt, R.W. Briddon, P.G. Markham, S. Oluwafemi, O.P. Windram, P. Lefeuvre, J.-M. Lett, D.P. Martin, Recombination, decreased host specificity and increased mobility may have driven the emergence of *Maize streak virus* as an agricultural pathogen. J. Gen. Virol. **89**, 2063–2074 (2008)
14. E. Moriones, J. Navas-Castillo, Tomato yellow leaf curl disease epidemics, in *Bemisia: Bionomics and Management of a Global Pest*, ed. by P.A. Stansley, S.E. Naranjo (Springer, Dordrecht, Heidelberg, London, New York, 2012), pp. 259–282
15. L. Amrao, S. Akhter, M.N. Tahir, I. Amin, R.W. Briddon, S. Mansoor, Cotton leaf curl disease in Sindh province of Pakistan is associated with recombinant begomovirus components. Virus Res. **153**, 161–165 (2010)
16. E.V.D. Walt, E.P. Rybicki, A. Varsani, J.E. Polston, R. Billharz, L. Donaldson, A.L. Monjane, D.P. Martin, Rapid host adaptation by extensive recombination. J. Gen. Virol. **90**, 734–746 (2009)
17. B.L. Patil, N. Dutt, R.W. Briddon, S.E. Bull, D. Rothenstein, B.K. Borah, I. Dasgupta, J. Stanley, H. Jeske, Deletion and recombination events between the DNA-A and DNA-B components of Indian cassava-infecting geminiviruses generate defective molecules in *Nicotiana benthamiana*. Virus Res. **124**, 59–67 (2007)
18. L.-F. Chen, M. Rojas, T. Kon, K. Gamby, B. Xoconostle-Cazares, R.L. Gilbertson, A severe symptom phenotype in tomato in Mali is caused by a reassortant between a novel recombinant begomovirus (*Tomato yellow leaf curl Mali virus*) and a betasatellite. Mol. Plant Pathol. **10**, 415–430 (2009)
19. A.P. Graham, D.P. Martin, M.E. Roye, Molecular characterization and phylogeny of two begomoviruses infecting *Malvastrum americanum* in Jamaica: evidence of the contribution of interspecies recombination to the evolution of malvaceous weed-associated begomoviruses from the Northern Caribbean. Virus Genes **40**, 256–266 (2010)
20. A. Kumar, J. Kumar, J.A. Khan, Sequence characterization of cotton leaf curl virus from Rajasthan: phylogenetic relationship with other members of geminiviruses and detection of recombination. Virus Genes **40**, 282–289 (2010)
21. P. Kumari, A.K. Singh, V.K. Sharma, B. Chattopadhyay, S. Chakraborty, A novel recombinant tomato-infecting *Begomovirus* capable of transcomplementing heterologous DNA-B components. Arch. Virol. **156**, 769–783 (2011)
22. J.K. Brown, K. Mills-Lujan, A.M. Idris, Phylogenetic analysis of *Melon chlorotic leaf curl virus* from Guatemala: another emergent species in the *Squash leaf curl virus* clade. Virus Res. **158**, 257–262 (2011)
23. J. Park, S. Kim, E. Choi, H.R. Kwak, M.K. Kim, K.Y. Lee, H.S. Choi, S. Lee, Molecular characterization of *Sweet potato leaf curl virus* (SPLCV) isolates from Korea: phylogenetic relationship and recombination analysis. Acta Virol. **55**, 327–335 (2011)
24. J. Park, H. Lee, M.K. Kim, H.R. Kwak, C.-K. Auh, K.-Y. Lee, S. Kim, H.-S. Choi, S. Lee, Phylogenetic lineage of *Tobacco leaf curl virus* in Korea and estimation of recombination events implicated in their sequence variation. Virus Res. **159**, 124–131 (2011)