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Both Indian cassava mosaic virus and Sri Lankan cassava mosaic virus are found in India and exhibit high variability as assessed by PCR-RFLP

Brief Report

B. L. Patil, S. Rajasubramaniam, C. Bagchi, and **I. Dasgupta**

Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi, India

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Summary. The biodiversity of geminiviruses associated with the Cassava Mosaic Disease (CMD) in India was investigated using PCR to specifically amplify the DNA of Indian cassava mosaic virus (ICMV) or Sri Lankan cassava mosaic virus (SLCMV) and also by using PCR to amplify specific viral genes, followed by digestion with different restriction endonucleases to obtain polymorphic patterns (PCR-RFLP). Results showed that both ICMV and SLCMV were present in mosaic-affected cassava; ICMV was geographically restricted to certain regions, whereas SLCMV was widespread. PCR-RFLP analysis showed that, in addition to ICMV-type and SLCMV-type patterns, a high proportion (40%) of the samples displayed novel patterns, some of which were localized in certain areas, whereas others were widely distributed.

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Cassava (*Manihot esculenta* Crantz, *Euphorbiaceae*) is grown for its starch-containing tubers, which feed over 500 million people worldwide and is the third most important food crop after cereals and grain legumes [9]. In India it is grown in an area of 2.4×10^5 hectares both for direct consumption and the starch grain (Sago)-producing industries, mainly in the southern states of Andhra Pradesh, Kerala and Tamil Nadu. The major constraint for cassava production in Africa and the Indian subcontinent [6, 10, 16–18] is the Cassava Mosaic Disease (CMD) caused by viruses included in the genus *Begomovirus* (family *Geminiviridae*). The genomes of most geminiviruses are bipartite, termed DNA A and DNA B, the former encodes functions associated with viral replication and encapsidation and the latter encoding the movement functions [5].

In Africa, CMD is caused by a number of begomoviruses representing the distinct species, such as *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus*, *East African cassava mosaic Zanzibar virus* and *South African cassava mosaic virus* [1, 3]. The causative agent of CMD in India is believed to be *Indian cassava mosaic virus*, ICMV [6, 11, 18]. Complete nucleotide sequencing of two cloned ICMV DNAs, one from the southern state of Kerala [6] and another from the central state of Maharashtra [15], showed that they were highly similar to each other, indicating them to be isolates of the same virus. In contrast, another distinct cassava-infecting geminivirus (CIG) was reported from Sri Lanka, named *Sri Lankan cassava mosaic virus* (SLCMV), which had much lower sequence homology to ICMV [15]. SLCMV had properties of a monopartite begomovirus, which reportedly captured the DNA B of ICMV following a recombination event [15]. A recent study involving sequence analysis of a few cloned CIG DNAs from India indicated some of them to be SLCMV and some to be ICMV (unpublished observations). The present study was undertaken to investigate the relative field distribution of ICMV and SLCMV and the diversity of CIGs in India. Using Polymerase Chain Reaction (PCR) analysis to specifically amplify parts of ICMV and SLCMV DNA A, we demonstrate, for the first time, the presence of both ICMV and SLCMV DNA in CMD-affected cassava plants from different field locations in India. In addition, by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis, we show the presence of several novel forms of the above viral DNAs, whose partial sequence analyses indicate that they have probably arisen by accumulating random point mutations.

To investigate the biodiversity of CIGs, samples were collected in the years 2001, 2002 and 2003, as stem cuttings from symptomatic cassava plants growing in fields from three states of southern India, namely Andhra Pradesh, Kerala and Tamil Nadu. Sampling was done from a total of 44 sites (Table 1). Stem cuttings were planted and the sprouted plants were maintained in a glass house at $30 \pm 2\,^{\circ}C$ with 16 hour light and 8 hour dark periods. For detecting the presence of CIG, nucleic acids were extracted from glasshouse-grown symptomatic cassava leaves using hot SDS method [2] and were blotted on to nylon membrane and screened for the presence of CIG by dot blot analysis [12] using a PCR-amplified ICMV DNA A fragment, encompassing the coat protein gene *AV*1 as the probe. The above probe showed a homology of 96.7% between ICMV and SLCMV, thus acting as a universal probe for CIGs in India. To differentiate between ICMV and SLCMV, total DNA from CMD-affected leaves were amplified using primers designed to distinguish ICMV from SLCMV. The forward primers were designed so as to specifically anneal either to the ICMV or to the SLCMV DNA A (2462–2481 for ICMV and 2690–2712 for SLCMV), whereas the common reverse primer could anneal to both the DNAs (614–633 for ICMV and 534–553 for SLCMV). PCR amplifications were carried out using both the discriminatory forward primers and the common reverse primer. Products of amplifications were analyzed on a 1% agarose gel with appropriate size markers.

Table 1. Distribution of cassava-infecting geminiviruses in different districts of southern India

District	Area under cassava cultivation (hectares)	Sample collection sites (no.)	No. of samples showing		
			ICMV	SLCMV	ICMV + SLCMV
Kerala	**112,774**				
Ernakulam	5,021	8	2	7	1
Idukki	7,027	1	1	0	0
Kollam	28,005	5	0	5	0
Kottayam	6,930	1	0	1	0
Kozhikode	4,182	8	16	3	1
Malappuram	8,043	1	0	1	0
Pathanamthitta	6,076	4	0	4	0
Thiruvananthapuram	26,631	4	0	7	0
Tamil Nadu	**74,121**				
Coimbatore	666	1	1	3	0
Salem	31,874	9	13	11	4
Andhra Pradesh	**17,694**				
East Godavari	16,781	2	0	6	0
Total		**44**	**33**	**48**	**6**

Source: Economics & Statistics Department, http://www.kerala.gov.in/index.htm; http://www.aphorticulture.com/tapioca_1.htm

For PCR-RFLP, primers were designed to amplify, from an average of 50 samples, the genes encoding Coat Protein, *AV*1 (between nucleotide residues 502–1281) and Replication-enhancer Protein, *AC*3 (1260–1738) from DNA A and Movement Protein *BC*1 (1189–2082) and Nuclear Shuttle Protein, *BV*1 (422–1210) from DNA B, based on the published nucleotide sequences of ICMV and SLCMV [15]. PCR-RFLP analysis was carried out using restriction endonucleases selected on the basis of their recognition sites to produce appropriate-sized fragments on gel electrophoretic separation. The amplified fragments were digested with restriction endonucleases according to the manufacturer's instructions and were analyzed by electrophoresis on 7% polyacrylamide gel, using standard methods [7].

When tested for the presence of CIG, a majority of samples tested positive by dot-blot hybridization with the probe. The probe hybridizes with both ICMV and SLCMV as they have a very high homology. It was not possible to distinguish between the presence of ICMV and SLCMV by the above method. To distinguish between ICMV and SLCMV, primers were designed to specifically amplify a 904 bp fragment from ICMV and a 599 bp fragment from SLCMV encompassing the 5′ portion of *AC*1 and the intergenic region. Thus, amplification reactions containing both the discriminatory and the common primers amplified a 904 bp fragment if only ICMV was present, a 599 bp fragment if only SLCMV was present and both the above fragments, if both the viruses were present. Out of a total of

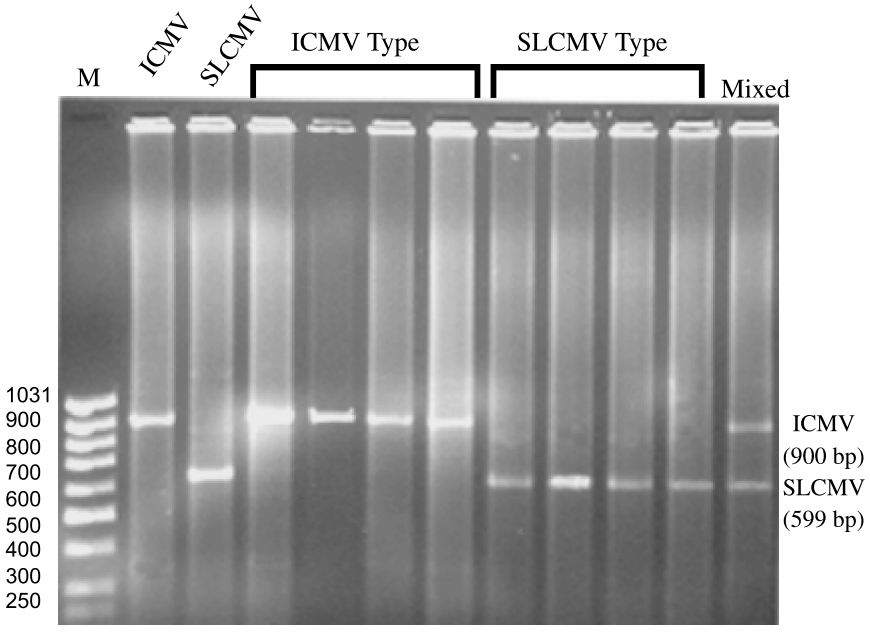

Fig. 1. PCR detection of ICMV and/or SLCMV DNA. Arrows at the sides indicate the expected positions of ICMV- and SLCMV-specific amplification products. Numbers at sides indicate the sizes of marker fragments in base-pairs

87 samples analyzed, 33 (38%) showed the presence of ICMV alone, 48 (55%) showed the presence of SLCMV alone and 6 (7%) showed the presence of both the viruses in them (Fig. 1 and Table 1). In the southern districts of Kerala (Kollam, Pathanamthitta and Thiruvananthapuram) SLCMV was the only virus detected, whereas in the northern districts (Kozhikode and Malappuram) both ICMV and SLCMV were present, ICMV being more predominant. In the central districts like Ernakulam, Kottayam and Idukki, both the viruses were present, SLCMV being more common. In the state of Tamil Nadu, both viruses were present to the same extent. Mixed infections with both the viruses were detected in a few samples from Tamil Nadu. CMD was observed only in a few fields in Andhra Pradesh and analysis of 6 such samples indicated the presence of SLCMV alone. Our results, which represent the overall variability of CIGs in India, indicate that ICMV was restricted to the northern and central districts of Kerala and to Tamil Nadu, whereas SLCMV was more widespread in all the CMD-affected regions surveyed, even in the few isolated CMD-affected pockets of Andhra Pradesh (Fig. 2).

RFLP patterns were observed in the four PCR-amplified DNA fragments, representing the various viral genes using two to six restriction enzymes. The patterns were divided into ICMV-type, SLCMV-type and novel (Table 2), predicted from the reported nucleotide sequences of the above viruses [15]. In some gene/enzyme combinations, for example *BC*1/*Sau* 3AI, no difference was expected in the RFLP patterns of the above two viruses and thus the patterns obtained were termed as either ICMV/SLCMV-type or novel. Fig. 3 illustrates the patterns obtained for the *BV*1 gene, displaying ICMV-type, SLCMV-type and several novel patterns. In the

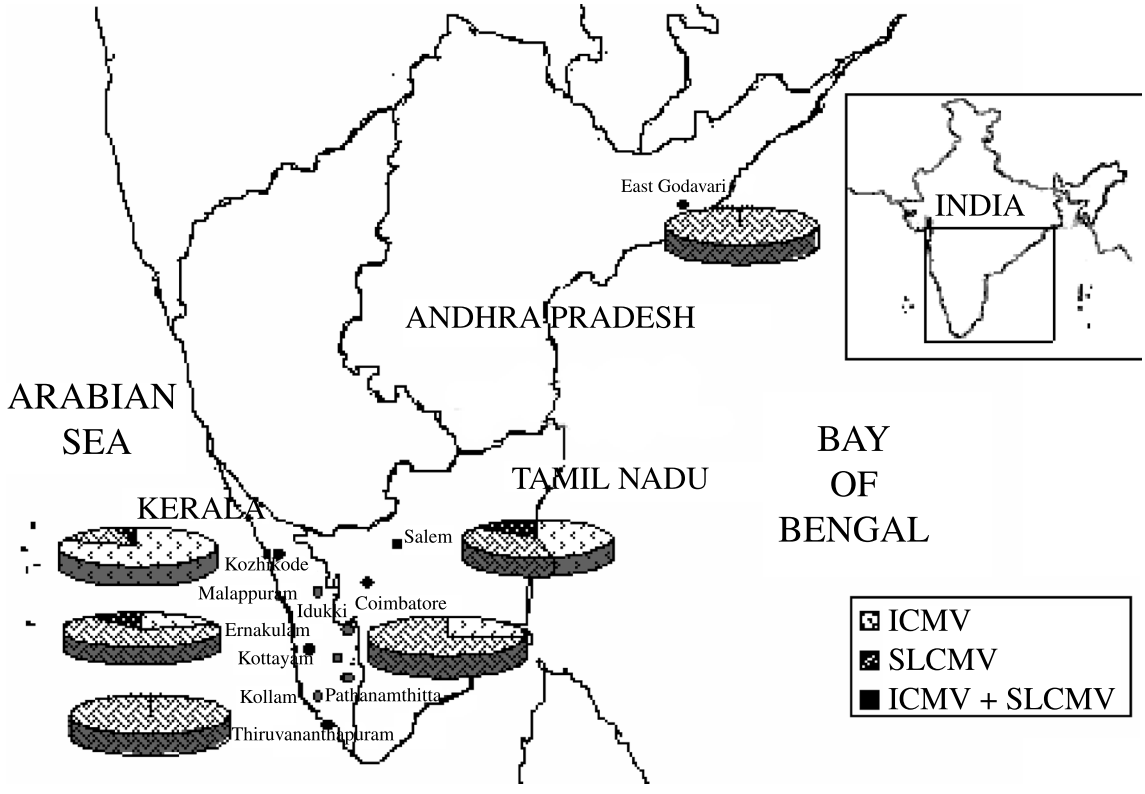

Fig. 2. Map of southern India indicating the proportion of ICMV, SLCMV and dual infection at various regions

5 geographical regions studied, between one to three novel RFLP patterns were observed, along with the expected ICMV-type, SLCMV-type and ICMV/SLCMV-type patterns (Table 2). The novel patterns were designated a, b and c types for each gene/restriction enzyme combinations. Some of the novel patterns were widespread whereas some others were restricted to specific regions. For example, the *AV*1/*Taq* I pattern "a" was widespread, but pattern "b" was observed only in northern Kerala and pattern "c" only in southern Kerala. Most samples displayed only one type of RFLP pattern, but there were few (less than 5%) which showed mixed infections with viruses showing both novel and ICMV-type or SLCMV-type pattern (Fig. 3). The proportion of samples showing novel patterns was very high in certain ORF/enzyme combinations, for example in *AC*3/*Sau* 3AI (90%) and *AV*1/*Taq* I (60%), but in most of the cases, it was found in roughly equal proportion to that of ICMV-type or SLCMV-type (Table 2). In order to determine whether random point mutations or possible recombination events were responsible for the novel RFLP types, nucleotide sequence information was obtained directly on two PCR-amplified DNA fragments of the *BV*1 gene representing different novel RFLP types derived from *Sau* 3AI digestion. Analysis of a stretch of 658 nucleotide residues (from 60th to the 718th residue from the ATG, representing start of *BV*1 gene) showed that there were 8 point mutations in the DNA representing RFLP

Table 2. Region-wise distribution and proportion* of the PCR-RFLP patterns representing ICMV, SLCMV and novel forms in cassava-infecting geminiviruses in India

Region		ORF/Restriction enzyme Pattern	AV1/*Taq* I	AV1/*Rsa* I	AC3/*Sau*3AI	BC1/*Rsa* I	BC1/*Sau*3AI	BC1/*Hinf* I	BC1/*Taq* I	BV1/*Sau*3AI	BV1/*Taq* I	BV1/*Rsa* I
Tamil Nadu		ICMV	−	++++	−	++	+++	+++	++++	+++	−	−
		SLCMV	+	+	−	−	−	+	−	−	−	++++
		Novel	++++	−	++++	+++	++	+	+	++	++++	+
		(Types)	(a)		(a,b)	(a,b)	(a)	(a,b)	(a,b)	(a)	(a,b)	(a,b)
Kerala	North	ICMV	−	++++	−	+++	+++	++	+++	+++	++	−
		SLCMV	++	−	+	−	−	−	++	+	+	+++
		Novel	+++	++	++++	++	++	++	−	+	+++	++
		(Types)	(a,b)	(b)	(a)	(a,b)	(a,b)	(a,b,c)		(a,c)	(a,c)	(a)
	Central	ICMV	−	++++	−	+	+++	+++	++++	+	+++	−
		SLCMV	+++	−	−	−	−	+	−	++	+	++++
		Novel	++	−	++++	++++	++	+	−	++	+	+
		(Types)	(a)		(a,b)	(a,b)	(b)	(b)		(b)	(b)	(a)
	South	ICMV	−	+	−	−	++	++++	++++	++	++	−
		SLCMV	+	++++	+	−	−	−	−	+	++	++++
		Novel	++++	−	++++	++++	+++	+	−	+++	+	+
		(Types)	(a,c)		(a)	(a,b)	(b)	(a)		(b,c)	(b)	(a)
Andhra Pradesh		ICMV	−	+	−	−	++++	+	++++	+	++	−
		SLCMV	+++	−	−	−	−	−	−	+++	−	++++
		Novel	++	++++	++++	++++	−	+++	−	−	++	−
		(Types)	(a)	(a)	(a)	(a,b)		(a)			(b)	

*Proportion of each type
+ = 1–25%, ++ = 26–50%, +++ = 51–75%, ++++ = 76–100%, − = absent

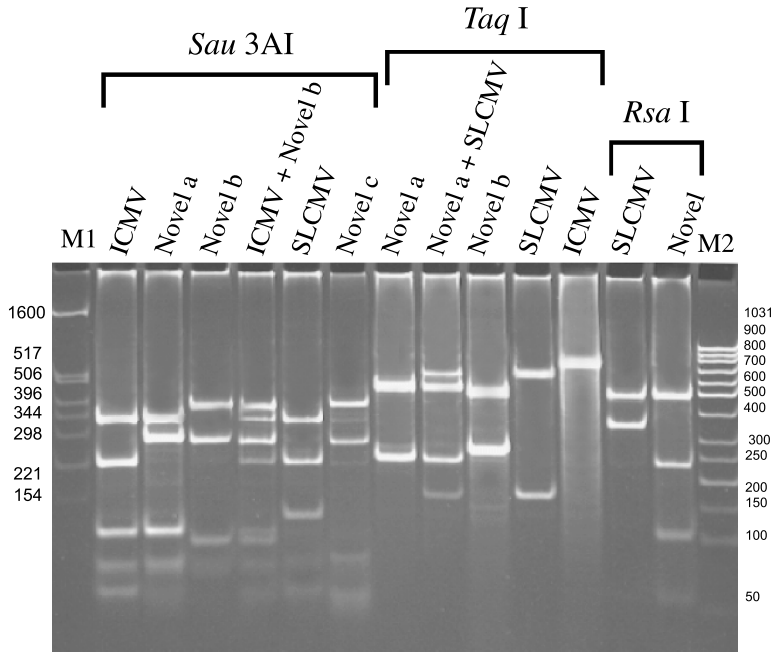

Fig. 3. PCR-RFLP patterns of Nuclear shuttle protein gene (*BV*1) from cassava DNA samples digested with *Sau*3AI, *Taq* I and *Rsa* I, two numbers indicating a mixed infection. Numbers at sides indicate size of the markers in base-pairs

pattern "a" and 10 in the DNA representing pattern "b", as compared to the ICMV and SLCMV sequences (data not shown). These mutations were seen to result in about equal number of amino acid substitutions, the new residues being mostly similar to the old one (data not shown). This indicated that, at least in the two novel RFLP-types studied, the polymorphism could be attributed to the presence of random point mutations, giving rise to novel RFLP patterns, and not to any clearly definable recombination event.

Properties of SLCMV, like increased aggressiveness, capability of capturing components of other viruses by recombination and infecting new hosts with satellite molecules like β [15] have important epidemiological implications for the spread of CMD and other geminiviral diseases. Using the method based on amplification of different sized DNA fragments representing ICMV A and SLCMV A, this analysis indicated that approximately half of the samples analyzed contained only SLCMV, a smaller portion contained only ICMV and a few samples contained both (Table 1). It appeared from the results that ICMV was found mainly in the northern-half of Kerala, with Ernakulam district forming the southern boundary and in Tamil Nadu. On the other hand, SLCMV was detected all throughout Kerala, Tamil Nadu and even in isolated pockets of CMD-infection in Andhra Pradesh. The more widespread distribution of SLCMV might be attributed to its more aggressive nature [15], to the genotypes of cassava grown in those regions, to the vector whitefly populations and possibly to germplasm movement. This observation on the regional restriction of CIGs in India is similar to that reported

in Uganda, where isolates of EACMV and ACMV were found to be restricted to certain regions of the country [13].

PCR-RFLP data on CIGs, generated from ten combinations of viral genes and restriction enzymes indicated that novel patterns were common in many samples (Table 2, Fig. 3). Similar observations have also been reported in other viruses, like plum pox potyvirus in Slovakia and rice tungro bacilliform virus in Philippines and India [8, 19], where it was suggested that these patterns represented "microvariants" of the major viral nucleic acid sequences, which have established themselves in certain regions. Probably the novel patterns reported in this study also represent a similar phenomenon in geminiviruses. The importance of these variants in the epidemiology of CMD in India awaits further investigation.

In Uganda, the severe outbreak of CMD in the early nineties has been attributed to both synergism and recombination between several strains of CIGs [4, 13, 20]. A similar situation has also been reported from Pakistan for geminiviruses of cotton [14]. The present study demonstrated that in India, field-grown cassava contains both ICMV and SLCMV, displaying high variability. Such a scenario can give rise to recombination or synergism between the existing viruses, resulting in a situation similar to the African CMD epidemic [13, 17, 18, 20]. The high proportion of SLCMV, which has already been shown to be capable of infecting newer species of plants and acquiring DNA components of other viruses [15] in the field, is a matter of concern. As a precautionary measure, it is necessary to intensify efforts to control CMD in India by monitoring germplasm movement between regions and by deploying resistant varieties of cassava by conventional or transgenic approaches.

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References

1. Berrie LC, Rybicki EP, Rey MEC (2001) Complete nucleotide sequence and host range of *South African cassava mosaic virus*: Further evidence of recombination amongst begomoviruses. J Gen Virol 82: 53–58
2. Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA mini-preparation Version II, Pl Mol Biol Rep 1: 19–21
3. Fauquet C, Fargette D (1990) African cassava mosaic virus: Etiology, epidemiology, and control. Plant Dis 74: 404–411
4. Fondong VN, Pita JS, Rey MEC, de Kockko A, Beachy RN, Fauquet CM (2000) Evidence of synergism between African cassava mosaic virus and a new double recombinant geminivirus infecting cassava in Cameroon. J Gen Virol 81: 287–297

5. Harrison BD, Robinson DJ (1999) Natural genomic and antigenic variation in whitefly-transmitted geminiviruses (begomoviruses). Ann Rev Phytopathol 37: 369–398
6. Hong YG, Robinson DJ, Harrison BD (1993) Nucleotide sequence evidence for occurrence of three distinct whitefly-transmitted geminiviruses in cassava. J Gen Virol 74: 2437–2443
7. Joshi R, Kumar V, Dasgupta I (2003) Detection of molecular variability in rice tungro bacilliform viruses from India using polymerase chain reaction-restriction fragment length polymorphism. J Virol Methods 109(1): 89–93
8. Kudela O, Glasa M, Fuchs E, Kudelova M (1998) Strain variability of plum Pox Virus isolates from Western Slovakia. Acta Virol 42: 71–74
9. Puonti-Kaerlas J (2001) Molecular biology of cassava. Hort Rev 26: 85–159
10. Malathi VG, Nair NG, Shanta P (1985) Cassava mosaic disease. Technical Bulletin Series 5, Central Tuber Crops Research Institute, Trivandrum, India, pp 18
11. Malathi VG, Thankappan M, Nair NG, Nambison B Ghosh SP (1987) Cassava mosaic disease in India. In: The International Seminar on African Cassava Mosaic Disease and its Control. Yamoussoukro, Cote d' Ivoire, 1987, CTA/FAO/ORSTOM/IITA/IAPC, pp 189–198
12. Maule AJ, Hull R, Donson J (1983) The application of spot hybridisation to the detection of DNA and RNA viruses in plant tissues. J Virol Methods 6: 215–224
13. Pita JS, Fondong VN, Sangare A, Otim-Nape GW, Ogwal S, Fauquet CM (2001) Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. J Gen Virol 82: 655–665
14. Sanz AI, Fraille A, Garcia-Arenal F, Zhou X, Robinson DJ, Khalid S, Butt T, Harrison BD (2000) Multiple infection, recombination and genome relationships among begomovirus isolates found in cotton and other plants in Pakistan. J Gen Virol 81: 1839–1849
15. Saunders K, Nazeera S, Mali VR, Malathi VG, Briddon R, Markham PG, Stanley J (2002) Characterisation of Sri Lankan cassava mosaic virus and Indian cassava mosaic virus: evidence for acquisition of a DNA B component by a monopartite begomovirus. Virology 293: 63–74
16. Swanson MM, Harrison BD (1994) Properties, relationships and distribution of cassava mosaic geminiviruses. Trop Sci 34: 15–25
17. Thresh JM, Otim-Nape GW, Legg JP, Fargette D (1997) African cassava mosaic virus disease: the magnitude of the problem. Afric. J Root Tuber Crops 2: 13–19
18. Thresh JM, Otim-Nape GW, Thankappan M, Muniyappa V (1998) The mosaic diseases of cassava in Africa and India caused by whitefly-borne geminiviruses. Rev Plant Pathol 77: 935–946
19. Villegas LC, Druka A, Bajet NB, Hull R (1997) Genetic variation of rice tungro bacilliform virus in the Philippines. Virus Genes 15: 195–201
20. Zhou X, Liu Y, Calvert L, Munoz C, Otim-Nape GW, Robinson DJ, Harrison BD (1997) Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J Gen Virol 78: 2101–2111

Author's address: Dr. I. Dasgupta, Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, New Delhi 11002, India; e-mail: indranil57@hotmail.com