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BRIEF REPORT

A new strain of *Indian cassava mosaic virus* causes a mosaic disease in the biodiesel crop *Jatropha curcas*

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Abstract *Jatropha curcas* mosaic disease is a newly emerging disease that challenges the productivity of a prospective biofuel crop, *J. curcas*. The aetiology of this disease has not been resolved. Here, we report the complete nucleotide sequences of a Jatropha virus isolated from Dharwad, Southern India. Phylogenetic analysis of the virus genome suggests it is a new strain of *Indian cassava mosaic virus*. Agroinfiltration of the two cloned viral DNA components produced systemic infection and typical mosaic symptoms in *J. curcas*, thereby fulfilling Koch's postulates. The availability of infectious clones will provide a valuable tool to screen *J. curcas* cultivars for disease resistance and facilitate the generation of virus-resistant *J. curcas* plants by transgenic technology.

Introduction

Jatropha curcas is a small woody plant belonging to the family Euphorbiaceae. Several unique characteristics of *J. curcas* plants make it suitable for biodiesel production. These include its rapid growth, easy propagation, low cost of seeds, high oil content, short gestation period, wide adaptability, drought tolerance and the ability to thrive on degraded soils [3, 6]. The latter characteristics are important, as they would allow the use of marginal or non-arable wasteland for the development of large-scale *J. curcas* plantations. However, *J. curcas* also suffers from several shortcomings that may constrain its wide adoption. For example, recently, *Jatropha curcas* mosaic disease (JcMD) has been found to seriously reduce productivity of *J. curcas* plants in the field [1]. The disease incidence was significant: about 25% in Northern India [11] and up to 47% in Southern India [1].

JcMD has been shown to be associated with a begomovirus based on virus transmission by the whitefly *Bemisia tabaci* (gennadius) and the partial coat protein (CP) sequence of the virus was reported [1]. However, the nature of the virus is unknown, and its phylogenetic relationship with other begomoviruses has not yet been established. Here, we report the cloning and characterization of the two DNA components of a *J. curcas* virus isolate. Inoculation of *J. curcas* with these infectious viral components produced typical JcMD symptoms, therefore fulfilling Koch's postulates. Further phylogenetic analysis showed that this *J. curcas* virus is a new strain of *Indian cassava mosaic virus* (ICMV), which is a major viral pathogen causing the cassava mosaic disease [12].

JcMD shows typical leaf symptoms of plants infected with geminivirus, including yellow-green mosaic, curling, malformation and size reduction. In addition, infected plants also displayed other symptoms such as a shortened internode and stunting of plant stature, and they produced few flowers and were partially or completely sterile (Fig. 1a, b). Leaves of mosaic-diseased *J. curcas* plants from Dharwad, Southern India, were collected in 2008. DNA extraction was performed using a DNeasy Plant Mini Kit (Qiagen). A partial DNA-A sequence was PCR-amplified in a 50 μL volume containing 1× PCR buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTP, 1 μg total DNA, 100 pmol each primer, 15% glycerol and 1.5 units Taq DNA polymerase (Temasek Life Science Laboratory)

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Table 1 Sequences of primers used in this report

Primer name	Sequence (5′–3′)
PA	TAATATTACCKGWKGVCCSC
PB	TGGACYTTRCAWGGBCCTTCACA
A-FF	CGTCAAGCTTGTATAATTGTGGTC
A-FR	ACAAGCTTGACGCAGCGTTCTGAG
B597F	AGACCTTATCGGAGGATCAA
B1062R	CTAGTCCAGATAGGGTATCT
B-FF	CAGTCGTCGTTTTCATGGGAAATA
B-FR	CCTTTGAAACGGCACATATGAT
B-NcoI F	CTCTCCATGGGTTTATGTCCTTCA
B-NcoI R	CATGCCATGGAGAGTATTGTACAG

with the universal primer pair PA/PB [3]. To clone full-length viral DNA-A, the reverse PCR primer pair A-FF/A-FR (Table 1) was further designed and used for amplification with Phusion High-Fidelity DNA Polymerase (Finnzymes). The full-length viral DNA-A was further cloned into pGEM-T Easy Vector (Promega), and full length sequences were generated from overlapping sequencing reactions using several primers designed to cover the whole DNA-A genome.

This *J. curcas* virus DNA-A genomic component encodes eight putative open reading frames (ORFs) (GenBank accession no: GQ924760). Only AV1 and AV2 are in the sense direction, whereas the other six ORFs are in the antisense direction. We performed pairwise sequence analysis of this *J. curcas* virus DNA-A with those of all known geminiviruses. The highest nucleotide sequence identity was found with the *Indian cassava mosaic virus* (ICMV) Ker2 strain (accession no.: AJ575819), which shares 92.4% identity with the Jatropha virus DNA-A sequence. These data suggest that the *begomovirus* is a new strain of ICMV according to currently accepted criteria [5], and accordingly, we named the isolate *Indian cassava mosaic virus*-Dharwad (India: Dharwad: 2008) [ICMV-Dha(IN:Dha:08)]. The ICMV-Dha virus is distinct in sequence from ICMV isolates that have been recovered from diseased cassava. Whether this sequence divergence is due to host-specific adaptation requires sequence characterization of additional germiniviruses from *J. curcas*.

The viral DNA-B was amplified and sequenced using the same procedure and protocol as for DNA-A, but with different primers. After having discovered that the ICMV-Dha is a new strain of ICMV, we deduced that the DNA-B might also share higher nucleotide sequence similarity with that of ICMV. Accordingly, we designed PCR primers (B597F/B1062R, Table 1) based on all reported ICMV DNA-B full-length sequences to amplify a partial sequence of the ICMV-Dha DNA-B. Using appropriate primers, we also cloned the full-length viral DNA-B into pGEM-T Easy Vector (Promega). We determined the full-length sequences with overlapping sequencing reactions using several primers designed to cover the whole DNA-B genome.

We used pBA002 and pCAMBIA1300 vectors to generate infectious ICMV-Dha DNA-A and DNA-B constructs, respectively. All viral DNA regions amplified by PCR were reconfirmed by direct sequence analysis. We used primer pair A-FF/A-FR to amplify the full-length sequence of ICMV-Dha DNA-A with a *Hin*dIII site at each end of the 1.0A product. Subsequently, the 1.0A fragment was digested with *Hin*dIII and *Nco*I and inserted into the *Hin*dIII and *Nco*I sites of pBA002 to produce pBA-0.5A. After dephosphorylation, the *Hin*dIII-digested pBA-0.5A was ligated with the partially *Hin*dIII-digested 1.0A to produce a clone of ICMV-Dha DNA-A, pBA-1.5A, for infectivity assays. To construct a DNA-B clone for similar purposes we used primer pair B-*Nco*IF/B-*Nco*IR to amplify the full-length sequence of ICMV-Dha DNA-B with an *Nco*I site at each end of the 1.0B product. Subsequently, 1.0B was digested with *Eco*RI and *Nco*I and inserted into the *Eco*RI and *Nco*I sites of pCAMBIA1300 to produce p1300-0.8B. After dephosphorylation, the *Nco*I-digested p1300-0.8B was ligated with the *Nco*I-digested 1.0B to produce a clone of DNA-B, p1300-1.8B. Products of all sequencing reactions were resolved using an ABI PRISM 3770 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

pBA-1.5A and p1300-1.8B were introduced into *Agrobacterium* strain AGL1 by electroporation. Agrobacterial cells were grown, collected and resuspended in MMA (10 mM MES, 10 mM $MgCl_2$, 200 μM AS) solution to a final OD_{600} of 1.5. Agrobacterial suspensions carrying either pBA-1.5A or p1300-1.8B were mixed in equal volumes. The mixed culture was used to agroinfiltrate leaves of 2–3-week-old *Nicotiana benthamiana* plants with a 1 ml needleless syringe. Symptoms were recorded periodically. In three independent experiments, 19 of 20, 10 of 10, and 18 of 20 infiltrated *N. benthamiana* plants became infected. Typical JcMD symptoms, such as plant stunting, downward leaf curling and yellow-green mosaic leaves, first appeared at 10–14 days post-inoculation (dpi) (Fig. 1c). To infect *J. curcas*, whole plants were submerged in agrobacterial inoculuma and subjected to 80–90 kPa vacuum for 5 min, and the vacuum was then quickly released to allow the inoculum to rapidly enter plant tissues. Vacuum-infiltrated plants were grown in a growth chamber at 25°C with a 16 h light/8 h dark photoperiod cycle. In three independent experiments, 4 of 6, 3 of 6, and 5 of 6 of treated *J. curcas* plants became infected and showed symptoms similar to those of JcMD-infected Jatropha plants seen in the field. These symptoms included downward leaf curling, yellow-green mosaic, serration and leaf-size reduction, and other symptoms such as shortened

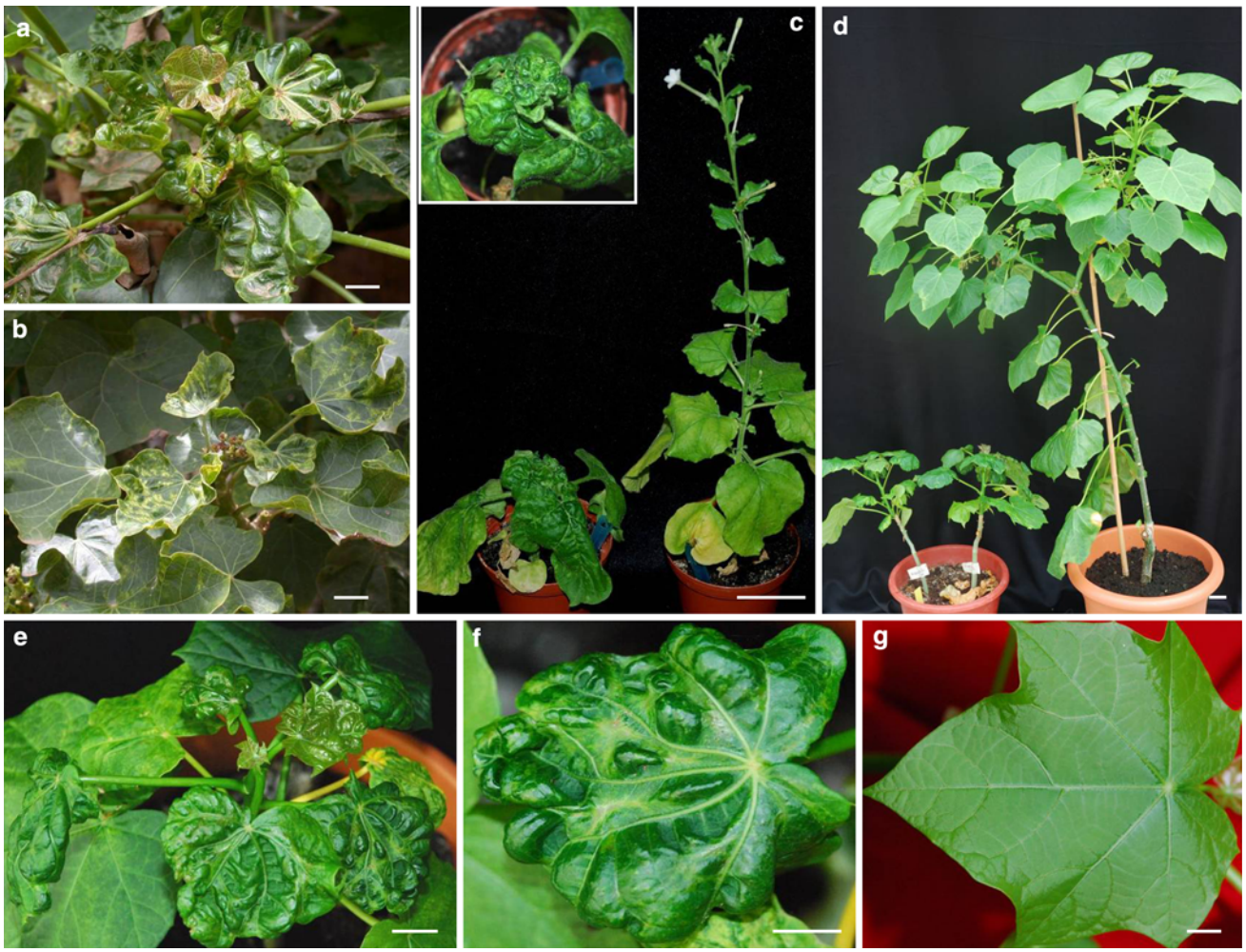

Fig. 1 Symptoms of *J. curcas* infected with the Jatropha virus in the field (**a, b**) or agroinfiltrated with ICMV-Dha DNA-A and -B clones (**d**–**f**) and symptoms of *N. benthamiana* plants infected by agroinfiltration of the same two clones (**c**). **a** An early infected plant with typical mosaic and malformed leaves symptoms on all the leaves. **b** A late infected plant with yellow-green mosaic on leaves and abscission of flowers and sterility. **c** Phenotypes of *N. benthamiana* 28 dpi infiltrated with ICMV-Dha DNA-A and –B constructs (left) and mock control (right). The inset shows the top view of treated plants with typical symptoms like stunting, downward leaf curling and yellow-green mosaic. **d** A *J. curcas* plant agroinfiltrated with ICMV-Dha DNA-A and DNA-B constructs for infectivity (left pot) and a healthy control plant (right pot). **e** *J. curcas* plant agriinfiltrated with ICMV-Dha DNA-A and -B clones showing symptoms similar to those of field infected *J. curcas* plants. Symptoms include yellow-green mosaic leaves, leaf serration, malformed leaves and stunting. **f** A higher-magnification view of a typical leaf displaying symptoms of ICMV-Dha infection. **g** Leaf of a healthy *J. curcas* control plant

internode and plant stunting were also observed (Fig. 1d, e). Early infection caused *J. curcas* plants to produce no flowers, and the infected plants were sterile (data not shown). Using primers specific for ICMV-Dha DNA-A and DNA-B, we confirmed systemic infection in both *N. benthamiana* and *J. curcas* (data not shown). These results provide conclusive evidence that the *begomovirus* is indeed responsible for JcMD in Southern India.

The complete nucleotide sequences of the infectious ICMV-Dha cloned components DNA-A and DNA-B are 2,735 and 2,724 nucleotides in length, respectively, and the genes are organised in a manner similar to those of other bipartite *begomoviruses* of the Old World [4] (GenBank accession no: GQ924760 and GQ924761) (Fig. 2). The complete DNA-A nucleotide sequence of ICMV-Dha is most similar to those of ICMV-Ker [IN:Ker2:02] (92.4%), ICMV-IN [IN:Mah:88] (90.9%) and ICMV-IN [IN:Mah2:88] (90.1%). Similar results were obtained when comparing individual ORFs amongst the DNA-A genomic components (Table 2). Systemic infection in *N. benthamiana* and *J. curcas* required the presence of both DNA-A and DNA-B, resembling the behaviour of ICMV in *N. benthamiana*, but differing from that of the *Sri Lankan cassava mosaic virus* (SLCMV). In the latter case, a single DNA-A can cause systemic infection and elicit obvious phenotypes in *N. benthamiana* and *Nicotiana glutinosa*

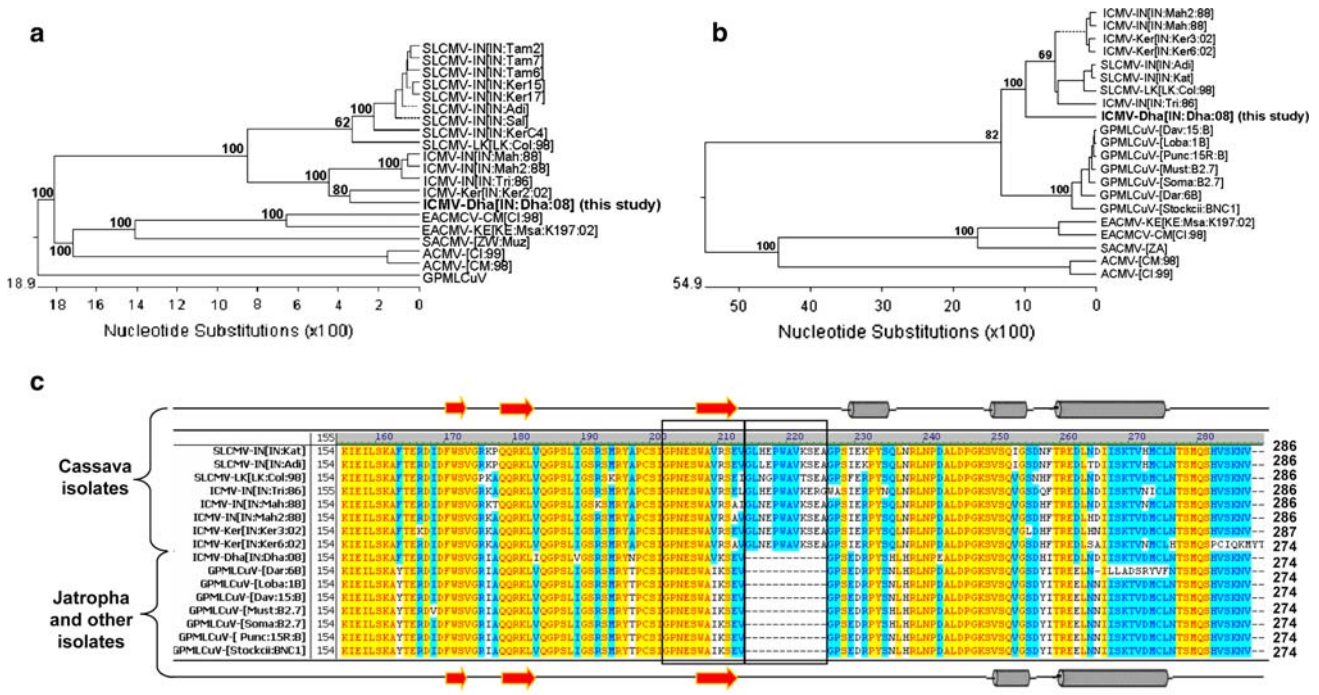

Fig. 2 Sequence analysis of ICMV-Dha genome and movement protein. Multiple sequence alignments of full-length DNA-A, DNA-B, and MP were carried out using the ClustalV program (MegAlign, DNAStar) with default parameters. Phylogenetic trees were constructed from multiple alignments using the neighbor-joining majority-rule consensus. Multiple alignments were analyzed by maximum parsimony with full-length DNA-A and DNA-B phylogenetic trees using Phylogenetic Analysis Using Parsimony (PAUP), and a bootstrap analysis with 1000 replicates was performed. Only values above 50% are reported on the trees in the figures. **a** Phylogenetic tree showing the sequence relationship between DNA-A of ICMV-Dha [IN:Dha:08] (GQ924760) and those of other cassava related mosaic geminiviruses. ACMV, African cassava mosaic virus; EACMCV, East African cassava mosaic Cameroon virus; EACMV, East African cassava mosaic virus; SACMV, South African cassava mosaic virus; ICMV, Indian cassava mosaic virus; SLCMV, Sri Lankan cassava mosaic virus; GPMLCuV, Gossypium punctatum mild leaf curl virus. Accession numbers are: SLCMV-IN [IN:Tam2], AJ890227; SLCMV-IN [IN:Tam7], AJ890229; SLCMV-IN [IN:Tam6], AJ890228; SLCMV-IN [IN:Ker15], AJ890224; SLCMV-IN [IN:Ker17], AJ890225; SLCMV-IN [IN:Adi], AJ579307; SLCMV-IN [IN:Sal], AJ607394; SLCMV-IN [IN:KerC4], AJ890226; SLCMV-LK [LK:Col:98], AJ314737; ICMV-IN [IN:Mah:88], AJ314739; ICMV-IN [IN:Mah2:88], AY730035; ICMV-IN [IN:Tri:86], Z24758; ICMV-Ker [IN:Ker2:02], AJ575819; EACMCV-CM [CI:98], AF259896; EACMV-KE [KE:Msa:K197:02], AJ717555; SACMV-[ZW:Muz], AJ575560; ACMV-[CI:99], AF259894; ACMV-[CM:98], AF112352; GPMLCuV, EU365617. **b** Phylogenetic tree showing the sequence relationship between DNA-B of ICMV-Dha [IN:Dha:08] (GQ924761) and those of other cassava-related mosaic geminiviruses. Accession numbers are: ICMV-IN[IN:Mah2:88], AY730036; ICMV-IN [IN:Mah:88], AJ314740; ICMV-Ker[IN:Ker3:02], AJ575820; ICMV-Ker [IN:Ker6:02], AJ512823; SLCMV-IN [IN:Adi], AJ579308; SLCMV-IN [IN:Kat], AJ575821; SLCMV-LK [LK:Col:98], AJ314738; ICMV-IN [IN:Tri:86], Z24759; GPMLCuV-[Dav:15:B], EU384577; GPMLCuV-[Loba:1B], FJ218488; GPMLCuV-[Punc:15R:B], EU384578; GPMLCuV-[Must:B2.7], FJ218489; GPMLCuV-[Soma:B2.7], FJ218490; GPMLCuV-[Dar:6B], EU384576; GPMLCuV-[Stockcii:BNC1], FJ218491; EACMV-KE[KE:Msa:K197:02], AJ704973; EACMCV-CM[CI:98], AF155807; SACMV-[ZA], AF259897; ACMV-[CM:98], AF112353; ACMV-[CI:99], AF259895. **c** Sequence alignment of the MP to show a 12-amino-acid duplication event that had occurred in the ICMV/SLCMV clade. Abbreviations and accession numbers are listed in **b**. Protein secondary structure prediction was performed by using http://bioinf.cs.ucl.ac.uk/psipred. The positions in the multiple alignment of MP are indicated on top of the figure. Arrows indicate β-sheet, cylinders depict α-helical structures and lines represent coiled regions. The duplicated region is underlined with dark lines

[13]. There is no other high sequence similarity region besides the common regions (CR) between the DNA-A and DNA-B genomic components. Iteron sequences (GGTACTCA separated by 21 nucleotides), which have been implicated in the binding of the replication-associated protein, are identical between ICMV and other cassava-infecting ICMV Ker and Mah isolates [12]. Although the iterons of the ICMV are different from those of SLCMV, we obtained infectious pseudorecombinants in *N. benthamiana* that presumably arose by reassortment of the cloned components of ICMV and SLCMV (data not shown). The three 41-nucleotide repeats within the common region of the original ICMV isolate (accession numbers Z24758 and Z24759) are absent in ICMV-Dha [6].

We found that two ORFs in the ICMV-Dha DNA-B genomic component show the highest amino acid sequence identity with that of *Gossypium punctatum mild leaf curl virus* (GPMLCuV). In particular, we note that the ICMV-Dha DNA-B genome, as well as that of the GPMLCuV, lacks a 36-nucleotide region when compared with the

Table 2 Genomic DNA and protein amino acid identities (%) between ICMV-Dha and other closely related begomoviruses

Begomoviruses	Genomic DNAs		Protein amino acid							
	DNA-A	DNA-B	AC1	AC2	AC3	AC4	AV1	AV2	MP	NSP
ICMV-IN [IN:Mah:88]	91	**85**	**94**	**93**	85	90	93	88	89	86
ICMV-IN [IN:Mah2:88]	90	83	92	92	87	90	92	88	89	86
ICMV-Ker [IN:Ker3:02]	NA	**85**	NA	NA	NA	NA	NA	NA	89	87
ICMV-Ker [IN:Ker6:02]	NA	83	NA	NA	NA	NA	NA	NA	88	87
ICMV-IN [IN:Tri:86]	85	75	91	91	86	87	92	86	85	88
ICMV-IN [IN:Ker2]	**92**	NA	**94**	NA	**91**	**92**	**95**	**93**	NA	NA
SLCMV-LK [LK:Col:98]	79	82	77	89	85	50	63	84	88	88
GPMLCuV-[Stockcii:BNC1]	NA	74	NA	NA	NA	NA	NA	NA	**94**	**89**

Highest values are indicated in bold letters

NA, sequence not available

DNA-B genomes of all other isolates of the ICMV/SLCMV clade. This region encodes a 12-amino-acid fragment at the *C*-terminus of the movement protein (MP), which is also a virulence factor [7]. The *C*-terminal region of MP is known to be important for its subcellular localization as well as viral movement and virus pathogenicity [7]. It appears that a duplication event occurred in this region of ICMV/SLCMV in infected cassava after they diverged from ICMV-Dha/GPMLCuV (Fig. 2c, d). We performed secondary-structure predictions for all of the MPs of SLCMV, ICMV, and GPMLCuV. Interestingly, an additional α-helical structure was found near the 12-amino-acid duplication site in MPs of viruses isolated from cassava (Fig. 2c). This third α-helix might help to stabilize the MP or form a complex structure with the other two α-helices to bind DNA/RNA, as has been reported for a3 of the flock house virus B2 suppressor and the 2b suppressor of cucumber mosaic virus [2, 15]. It might also help to interact with cassava host factor or other geminivirus component such as nuclear shuttle protein to assist intracellular and systemic movement of the virus. The biological function of the extra 12-amino-acid segment of the MP of cassava-infecting geminiviruses is being investigated.

Originally from South/Central America, *J. curcas* is an introduced species in many Asia and African countries. This is similar to cassava, which was also introduced into Asia and Africa and suffers from *begomovirus* infection. In view of the worldwide interest in biofuel crops, many Asian countries have begun to develop large-scale *J. curcas* plantations to provide for biodiesel feed stock. The possibility of JcMD as a new epidemic disease poses a serious and increasing challenge to the Jatropha industry, because this virus reduces yield and could easily spread to plantations presently uninfected by the virus. The availability of cloned genomic components of ICMV-Dha that are infectious to *J. curcas* represents an important development to screen existing and new *J. curcas* varieties for disease resistance. Meanwhile, Jatropha transgenic technology has been established [8]. With the genome sequence information and the availability of the two infectious clones, it may be possible to use double-stranded hairpin RNA [14] or artificial miRNA-mediated RNA interfering technology [9, 10] to generate transgenic *J. curcas* lines that are resistant to ICMV-Dha.

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