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RESEARCH

Molecular Studies on the Transmission of Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV) in Cassava by *Bemisia tabaci* and Cloning of ICMV and SLCMV *Replicase* Gene from Cassava

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Abstract Cassava mosaic disease, caused by cassava mosaic geminiviruses are transmitted by *Bemisia tabaci*. The *B. tabaci* adults from colonies reared on virus free cassava plant produced from apical meristem culture was studied to determine their ability to transmit Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV) from cassava to cassava. Virus free plants were confirmed by polymerase chain reaction (PCR) using geminivirus degenerate primers. The virus acquisition access period (AAP) of 48 h on virus infected cassava leaves and 48 h virus inoculation access periods on virus free healthy leaves were investigated. Both ICMV and SLCMV were absolutely transmitted by whiteflies reared on cassava. Virus specific primers were designed in the *replicase* region and used to detect virus in *B. tabaci* after different AAP. The PCR amplified *replicase* genes from virus transmitted cassava leaves were cloned the plasmid DNA was isolated from a recombinant colony of *E. coli* DH5α after their confirmation by colony PCR and sequenced them. The nucleotide sequences obtained from automated DNA sequencing were confirmed as ICMV and SLCMV *replicase* gene after homology searching by BLAST and found to be a new isolates. The nucleotide sequences of new isolates were submitted in GenBank (accession number JN652126 and JN595785).

Keywords *B. tabaci* · Cloning · Meristem culture · Sequencing · Virus transmission

Abbreviations

AAP	Acquisition access period
ACMD	African cassava mosaic disease
ACMV	African cassava mosaic virus
BAP	*N*-6-Benzylaminopurine
Bp	Base pair
BLAST	Basic local alignment search tool
Cm	Centimetre
CMD	Cassava mosaic disease
CMGs	Cassava mosaic geminiviruses
CMV	Cassava mosaic virus
DNA	Deoxy ribonucleic acid
EACMCV	East African cassava mosaic Cameroon virus
EACMKV	East African cassava mosaic Kenya virus
EACMMV	East African cassava mosaic Malawi virus
EACMV	East African cassava mosaic virus
EACMZV	East African cassava mosaic Zanzibar virus
EtBr	Ethidium bromide
GA_3	Gibberellic acid
IAP	Inoculation access period
ICMV	Indian cassava mosaic virus
M	Molar

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µg	Microgram
ml	Millilitre
µl	Microliter
µM	Micromolar
mm	Millimeter
NCBI	National center for biotechnology information
ng	Nanogram
SACMV	South African cassava mosaic virus
SDS	Sodium dodecyl sulfate
SLCMV	Sri Lankan cassava mosaic virus
MS	Murashige and Skoog
MCM	Meristem culture medium
NAA	1-naphthalene acetic acid
PCR	Polymerase chain reaction
RH	Relative humidity
TNAU	Tamil Nadu Agricultural University

Introduction

Bemisia tabaci (Gennadius) (Homoptera: Aleyrodidae) is a serious agricultural pest in tropical and subtropical environments [7, 34]. In the past three decades, *B. tabaci* has invaded every continent in the world, with the exception of Antarctica. *B. tabaci* has been reported on more than 600 crop and weed hosts and it becomes increasingly important vectors of 111 recognized economically devastating plant viruses in the genera *Begomovirus* (Geminiviridae), *Crinivirus* (Closteroviridae), *Carlavirus* and *Ipomovirus* (Potyviridae) [18]. Geminiviruses (Family: Geminiviridae, Genus: *Begomovirus*) causing cassava mosaic disease (CMD) known as cassava mosaic geminiviruses (CMGs) transmitted by *B. Tabaci* have been reported from Africa and the Indian subcontinent [15].

There are nine begomoviruses namely, Indian Cassava Mosaic Virus (ICMV), Sri Lankan Cassava Mosaic Virus (SLCMV), African Cassava Mosaic Virus (ACMV), East African Cassava Mosaic Virus (EACMV), East African Cassava Mosaic Cameroon Virus (EACMCV), East African Cassava Mosaic Kenya Virus (EACMKV), East African Cassava Mosaic Malawi Virus (EACMMV), East African Cassava Mosaic Zanzibar Virus (EACMZV) and South African Cassava Mosaic Virus (SACMV) causing CMD in cassava. In India and Sri Lanka, the most important viral disease is known as Indian cassava mosaic disease (ICMD) and Sri Lankan cassava mosaic disease (SLCMD) respectively and is caused by one of nine begomoviruses, including ICMV [31, 39] and SLCMV. ICMD in India was first reported by Abraham [3] and is caused by ICMV or by SLCMV [33]. However, CMD have been reported in East Africa in 1894 itself [23]. *B. tabaci* is the only known insect vector of ICMV and SLCMV. Yield losses due to CMD of 20–90% have been reported in Worldwide [11, 14, 32, 40].

Recent years there are many reports of virus acquisition-transmission and host plant based biotypes in *B. tabaci*. Preliminary studies of the biochemical variability of *B. tabaci* on cassava and other crops in Trivandrum (Kerala, India), using isozyme analyses and host-associated variation studies provided evidence of at least two populations of *B. tabaci*. One population was adapted to cassava [26]. The cassava-adapted haplotype of *B. tabaci*, referred to as the Indian cassava biotype [26]. This study was initiated to investigate the ICMV and SLCMV transmission through *B. tabaci* from cassava to cassava and its confirmation by polymerase chain reaction (PCR), cloning and sequencing of *replicase* gene in order to find new isolates in Tamil Nadu. We conducted virus transmission studies under 48-h virus acquisition access (feeding) period (AAP) on virus infected cassava leaves and 48-h inoculation access (feeding) (IAP) period using *B. tabaci* population and later virus detection was done in the experimental cassava plant.

Materials and Methods

Virus Free Cassava Plants Through Apical Meristem Culture

The in vitro cassava variety H226 plants, developed at Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University (TNAU), were grown on meristem culture medium (MCM) consisting of Murashige and Skoog basal medium (MS) [29] supplemented with 0.1 mg/l 6-benzylaminopurine (BAP) plus 0.02 mg/l Gibberellic acid (GA_3). MCM was standardised for cassava variety H226 with nine different concentrations of BAP and GA_3 with five replications. The virus free cassava plants were produced according to Raghu et al. [35] by apical meristem culture. The explants were transferred to fresh medium every 7 days. When meristem tips developed a new stem were transferred to test tubes (25 × 150 mm) containing MCM. Plantlets consisting of a stems were eventually rooted in vitro on MCM. Three weeks after sub culturing, rooted plants were transferred to a hardening medium, plastic pots containing moistened sterilized coconut coir pith and the mouth of the pot along with plants were covered with polythene bag for increasing survival rate by maintaining the humidity. Then these potted plants were kept in shade net house for primary and secondary hardening with subsequent transferring of cassava plants for small size pot to bigger one. All the plants were watered weekly with a Hoagland solution [17] and when sufficiently mature were transferred into the greenhouse.

Regenerated cassava plants were detected for cassava mosaic virus (CMV) by visual inspection and PCR using degenerate primer pair [13] for geminiviruses. The DNA was extracted from the in vitro apical meristem derived cassava plants by SDS–potassium–acetate method [12]. The PCR amplification reactions were performed in a 20 µl volume using 50 ng of DNA containing 0.2 µM of each forward (5′-TAATATTACCKGWKGVCCSC-3′) and reverse (5′-TGGACYTTRCAWGGBCCTTCACA-3′) primer pair, 20 µM of dNTPs, 2 µl (1X) Taq polymerase buffer, 1.5 mM $MgCl_2$, and 0.3 U Taq DNA polymerase and the reaction was carried out using PTC100 (M/s. MJ Research Inc.) Thermal cycler program comprised of 5 min at 94 °C, followed by 40 cycles of 1 min at 94°C, 1 min 58°C, and 1 min at 72°C, and final extension time 10 min at 72°C. Then the amplified PCR products were verified for the presence/absence of virus by agarose gel electrophoresis. Infectious material as a positive control was always included in the virus detection.

Culturing of *B. tabaci* (whitefly)

The *B. tabaci* (CMV free) cassava strain was reared on cassava cultivar H226 or eggplant cultivar Pusa Kranti. Adult *B. tabaci* used to initiate the colony on eggplant were collected from cassava plants growing at TNAU Orchard, Coimbatore in 2011. Non-viruliferous *B. tabaci* colonies were established by transferring newly enclosed adult for several generations on a non-host ICMV and SLCMV eggplant [14], followed by colonization on virus free apical meristem derived cassava plants grown in an insect-proof cage were considered to be free of virus. The *B. tabaci* colonies were maintained by transferring, every 4–6 weeks, to a virus free plant placed in a cage in an insect free screen house. Cages were 70 × 42 × 42 cm and were covered with organdy cloth on three sides and transparent plastic sheets on the fourth side and on the top of the cage.

Culturing of CMV

Virus free apical meristem derived cassava plants were cultured in the net house as described earlier [5]. ICMV and SLCMV-infected young cassava leaves were pruned and young sprouts showing severe symptoms were ground with a pre-cooled mortar and pestle in 0.1 M phosphate buffer (pH 7.0). The crude extracts were inoculated into virus free apical meristem derived cassava plants dusted with 600 mesh carborundum/charcoal powder and subsequently kept in an insect proof greenhouse. Virus infected cassava plants were used as a source of inoculums for virus transmission experiment.

Transmission of CMV

General method for virus acquisition and transmission employed as described earlier by Antony et al. [5] with slight modifications [36]. For virus transmission studies, virus free cassava plants derived from apical meristem culture were grown in 21 cm diameter plastic pots containing sterilized soil (autoclave at 121°C with 15 psi (100 kPa) for 20 min) in insect-proof cages. Nutrient (Hoagland's) solution was added to the soil at 3-day intervals [17]. Twenty adult cassava strain whiteflies (non-viruliferous/CMV free) were collected from their respective colonies, starved for 3 h, and released into leaves of young symptomatic/virus infected seedlings. After 48 h AAPs, whiteflies were collected by aspiration and starved for 3 h. Thereafter, 20 cassava strain whiteflies were released into virus free (non-diseased or uninfected) healthy apical meristem derived cassava plants. Following 48 h IAPs, the insects were aspirated from the cages and killed. Plants were carefully removed from insect proof cages kept in the screen house (25–32°C and 65–70% RH) for 3 months of observation on the development of mosaic disease symptoms. The experiment was repeated on four occasions with five replications each.

PCR Detection of SLCMV and ICMV in *B. tabaci*

The effect of the length of the feeding period on ICMV and SLCMV acquisition by *B. tabaci* was determined by confining ten adult *B. tabaci* reared on virus free eggplant, after 3 h starvations, for 1, 2, 3, 5, 10, 24, 48 and 72 h AAP in young CMV infected cassava leaves. After each AAP, whiteflies were aspirated from plants and frozen at −30°C until PCR analysis. *B. tabaci* adults reared on eggplant (non-host of CMV) were negative controls. *B. tabaci* adults were homogenized in a sterile 1.5 ml microfuge tube with mini-drill (Eppendorf, Germany) using liquid nitrogen and transferred to a fresh sterile microfuge tube. The total DNA was extracted according to the Zeidean and Czosnek [41]. Standard PCR was carried out by using specific primers F: 5′-TGTGACCTTGATTGGCACCTG-3′; R: 5′-CTCGAC GAGTGGTTTCACGA-3′ for ICMV) and (F: 5′-TAGC TGCCCTGTGTTGGAC-3′; R: 5′-TGAGAAACCCACGA TTCAGA-3′ for SLCMV; designed based on the *replicase* gene sequence of both the viruses [36]. Reaction conditions were essentially those of Sambrook et al. [38]. PCR parameters were 94°C for 2 min then 40 cycles of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C, followed by the final extension of 10 min at 72°C. The amplified PCR products were checked on 1.5% agarose gel stained with 0.5 µg/ml of ethidium bromide and photographed.

Cloning of *Replicase* Gene

The *replicase* gene of ICMV and SLCMV were amplified through PCR using specific primer pairs from genomic DNA isolated from infected cassava plant and the product was resolved in 1.2% agarose gel. PCR product was column purified by PCR clean-up kit (Sigma-Aldrich, USA) according to manufacturer instructions. The purified products were ligated into pJET easy vector 1.2 (MBI Fermentas, USA). Competent cells of *E. coli* strain DH5α were transformed with the above ligated mixture and recombinant *E. coli* colonies were selected on LB plate with ampicillin (100 μg/ml). The transformed colonies were screened by colony PCR with screening primer pairs specific to *replicase* genes were designed [36]. PCR Reactions were performed using a PTC-100TM Programmable Thermal Controller (MJ Research, Inc., USA) with single colony of DH5α, in a 20 μl reaction volume by following the reaction mixture and temperature profiles were done as mentioned before. After amplification, 10 μl of the product was used for electrophoretic analysis on a 1.2% agarose gel. The plasmid DNA was isolated from recombinant colonies and sequenced.

Data Analysis

In this study, mean (five replications) and standard error were calculated using Microsoft office Excel 2007 and standard deviation, critical difference (CD) and coefficient of variation (CV) data were analyzed statistically by completely randomized designs (CRD) using Agres Statistical Software Version 3.01 [1]. The cloned ICMV and SLCMV *replicase* genes were sequenced by automated DNA sequencing. The nucleotide sequence of ICMV and SLCMV *replicase* genes were compared with other cassava infecting geminiviruses available in the publically available database NCBI using BLAST search and submitted in GenBank using Bankit tool.

Results

Apical Meristem Culture of Cassava Variety H226

Apical meristems were excised from in vitro cultured cassava shoots cultured in MCM, after 12 days in culture, meristems were healthy and chlorophyllous, moreover the shoot and root length and number of roots were also positively high significant with the regeneration frequency of 93.11%. When the complete regeneration of plants was achieved, plantlets some 6–8 cm tall were selected for transplantation. After rooting, the plants were hardened in culture room for a week before transferred to shade net house resulted in 100% survival. The effect of different concentration of GA_3 and BAP contained MCM on cassava variety H226 were given in the Table 1 and the in vitro plant regeneration from apical meristem cultures of Cassava variety H226 and in vitro rhizogenesis were given in the Fig. 1.

Detection of CMV in Apical Meristem Derived Plants

To confirm the absence/presence of CMV in meristem derived cassava plants of variety H226, DNA was isolated from the hardened plants maintained in the greenhouse. Then the isolated DNA from different plant samples was amplified in the MJ Research Thermal Cycler, where PCR was carried out using Deng's degenerate primer. The DNA from the CMV infected plant, with obvious mosaic symptom was amplified, for treating as check. Then, PCR product of the samples was electrophoreses on agarose gel. An amplified DNA fragment of about 560 bp was obtained, only from the DNA isolated from the plants of H226 cassava plants, which are supposed to carry the virus, while there was no amplification of 560 bp DNA fragment observed from the in vitro regenerated meristem derived plants of this variety (Fig. 2) which indicates all the plants were 100% free from CMV (Table 2).

Transmission of SLCMV and ICMV

The viruliferous *B. tabaci* adults were released on CMV free young cassava plants for 48 h to inoculate the SLCMV and ICMV. The typical mosaic symptoms were appeared on leaves at 25th day of after inoculation and 80.5% of the cassava plants were found to be the symptoms of CMD during the 3rd month period.

PCR Detection of SLCMV and ICMV in Whiteflies Following Different AAPs

B. tabaci adults allowed AAPs on infected cassava plants of 1, 2, 3, 4, 5, 6, 10, 12, 24 and 48 h followed by extraction of genomic DNA and PCR confirmed viral acquisition of the virus. A 460 and 1,056 bp of PCR amplified DNA fragments of ICMV and SLCMV were obtained respectively (Fig. 3a, b). No amplicon was observed in the negative control in which the DNA from non-viruliferous *B. tabaci* was used as the template in PCR analysis.

Cloning and Sequencing of *Replicase* Genes

Total genomic DNA isolated from infected cassava variety H226 was used as a template for amplification of *replicase* genes by PCR using a gene specific primer set. The eluted

Table 1 Effect of different concentration of GA_3 and BAP in meristem culture medium (MCM) on cassava variety H226

Media for shoot regeneration						Media for in vitro rooting					
Media	Plant growth regulator (mg/l)		Number of multiple shoots (nos)*	Shoot length (nos)*	No of days for shoot elongation*	Media	Plant growth regulator (mg/l)		No of roots (nos)**	Root length (cm)**	No of days for rooting**
	GA_3	BAP	(Mean ± SE)	(Mean ± SE)	(Mean ± SE)		GA_3	BAP	(Mean ± SE)	(Mean ± SE)	(Mean ± SE)
MCM1	0.01	0.1	1.1 ± 0.105^{d}	3.43 ± 0.145^{c}	15.67 ± 0.333^{c}	MCM10	0.02	0.1	4.37 ± 0.033^{d}	4.27 ± 0.176^{c}	12.0 ± 0.16^{c}
MCM2	0.01	0.5	3.0 ± 0.061^{d}	3.53 ± 0.067^{c}	12.33 ± 0.333^{b}	MCM11	0.02	0.5	4.73 ± 0.120^{d}	5.14 ± 0.088^{c}	10.0 ± 0.1846^{b}
MCM3	0.01	1.0	2.0 ± 0.06^{c}	3.46 ± 0.133^{c}	15.33 ± 0.882^{c}	MCM12	0.02	1.0	3.83 ± 0.410^{e}	4.77 ± 0.088^{d}	13.1 ± 0.324^{d}
MCM4	0.02	0.1	6.3 ± 0.099^{a}	5.26 ± 0.120^{a}	7.00 ± 0.577^{a}	MCM13	0.5	0.1	6.04 ± 0.088^{c}	5.87 ± 0.033^{c}	8.0 ± 0.113^{b}
MCM5	0.02	0.5	1.0 ± 0.035^{d}	3.47 ± 0.120^{c}	15.33 ± 1.202^{c}	MCM14	0.5	0.5	6.67 ± 0.088^{b}	6.07 ± 0.067^{b}	8.1 ± 0.182^{b}
MCM6	0.02	1.0	4.1 ± 0.226^{b}	3.87 ± 0.318^{c}	13.00 ± 0.577^{b}	MCM15	0.5	1.0	7.24 ± 0.176^{a}	6.73 ± 0.088^{a}	7.1 ± 0.398^{a}
MCM7	0.03	0.1	6.1 ± 0.412^{a}	5.16 ± 0.145^{a}	9.00 ± 0.577^{a}	MCM16	1.0	0.1	3.27 ± 0.088^{f}	3.33 ± 0.088^{d}	16.3 ± 1.1^{f}
MCM8	0.03	0.5	4.1 ± 0.059^{b}	4.53 ± 0.088^{b}	10.67 ± 0.667^{a}	MCM17	1.0	0.5	3.43 ± 0.120^{ef}	3.67 ± 0.120^{d}	13.3 ± 0.193^{e}
MCM9	0.03	1.0	3.0 ± 0.047^{c}	3.83 ± 0.120^{c}	13.00 ± 1.000^{b}	MCM18	1.0	1.0	3.57 ± 0.145^{ef}	3.97 ± 0.260^{d}	13.0 ± 0.203^{d}
		SED	0.5691	0.2511	0.8347			SED	0.2439	0.1826	0.6743
		CD (0.01)	1.7209	0.7335	2.7099			CD (0.01)	0.7023	0.5256	2.9371
		CD (0.05)	0.5832	0.5323	0.5512			CD (0.05)	0.5125	0.3836	0.5279
		CV %	7.78	7.85	7.83			CV %	6.24	4.59	6.79

Data shown are Mean ± SE of five replication, each experiment consisted of 20 cultures

Means followed by the *same letter* are not significantly different from each other

*, ** Measured at 29 and 38 days after inoculation of explants respectively

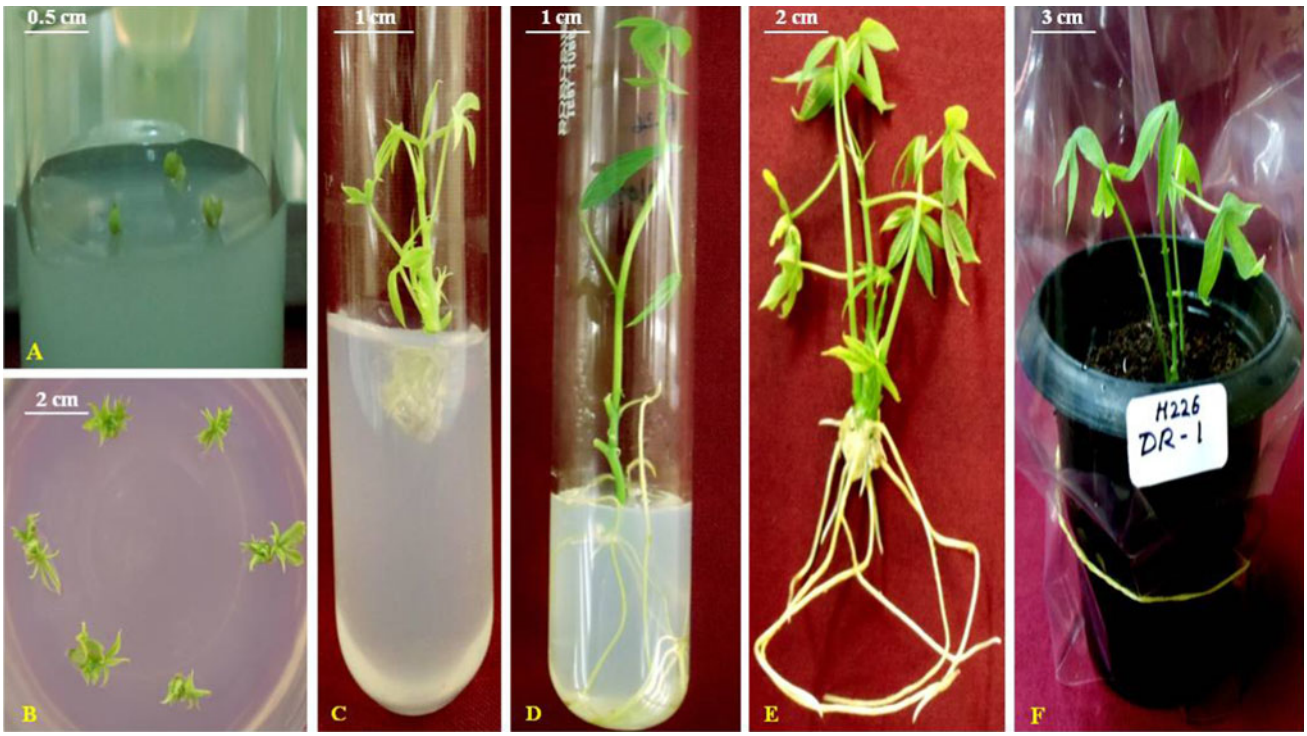

Fig. 1 In vitro plant regeneration from apical meristem cultures of Cassava variety H226 and induction of rhizogenesis. **a** One week old meristems on MCM; **b** 21 days old established or elongated meristems; **c** shoot induction from apical meristem; **d** induction of rhizogenesis on MCM15 after 5 weeks of culture; **e** complete plantlet; **f** hardening of in vitro raised plantlets maintained on hardening medium under greenhouse

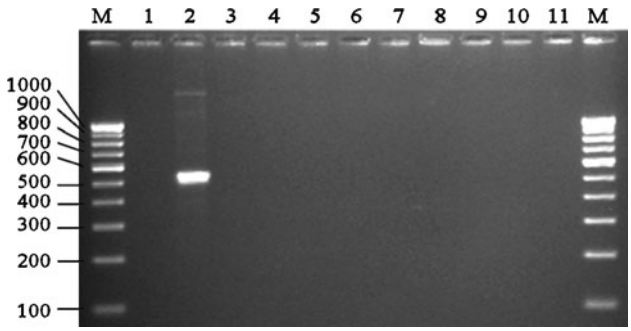

Fig. 2 Detection of cassava mosaic virus using PCR. *M* 100 bp ladder, *lane 1* negative control, *lane 2* positive control (PCR product of infected plant) and *lanes 3–12* PCR product of in vitro-raised meristem derived plants

replicase genes were ligated with pJET blund end cloning vector. The ligated mixture was transformed into competent cells of *E. coli* DH5α. The transformants were selected on LB plates containing ampicillin (100 µg/ml). Presence of insert was confirmed by colony PCR with screening primers (Fig. 4). Nucleotide sequences of ICMV and SLCMV cloned DNA fragment was obtained by automated DNA sequencing and submitted in the GenBank, NCBI after homology searching under the accession numbers JN652126 and JN595785 respectively using Bankit tool to available in the public database. The nucleotide sequences were compared with other cassava infecting geminiviruses in GenBank. Comparative sequence analyses showed that the cloned fragment shared a maximum sequence identity at nucleotide levels (99%) with respective viruses.

Discussion

This study illustrates the transmission of ICMV and SLCMV by *B. tabaci* from cassava to cassava and their corroboration through PCR, cloning and sequencing of specific *replicase* gene. In this experiment, the virus free, healthy cassava plants were produced through apical meristem culture [19, 20] for virus transmission.

Plant tissue culture has gained significance in recent years, particularly in rapid clonal propagation and production of pathogen free plants. Unlike fungal and bacterial diseases, which can be eradicated from crops by chemical sprays, the only practical method available for the eradication of viruses from vegetatively propagated plants is tissue culture [37]. The meristem explants have been chosen for the study, as Lal and Lal [21], reported that in the virus infected plant, the concentration of virus decreases acropetally towards the meristem of the apical bud. Virus movement is considerably slower through symplasm, than through the apoplastic vascular system.

Table 2 Effect of meristem culture on the cassava variety H226 plants tested negative for cassava mosaic virus (CMV)

Test	Percent explants showing established or elongated shoots	Length of shoots (nos)* (Mean ± SE)	Number of roots per shoot (nos)** (Mean ± SE)	Length of roots (cm)** (Mean ± SE)	% of well rooted shoots	% of CMGs negative plants
I in vitro	95.33	5.1 ± 0.145	7.3 ± 0.176	6.7 ± 0.088	97.84	100
II in vitro	90.67	5.5 ± 0.088	7.5 ± 0.145	6.6 ± 0.067	98.61	100
III in vitro	93.33	5.2 ± 0.120	6.9 ± 0.067	6.9 ± 0.176	96.6	100

Data shown are Mean ± SE of five replication, each replication consisted of 20 plants

*, ** Measured at 29 and 38 days after inoculation of explants respectively

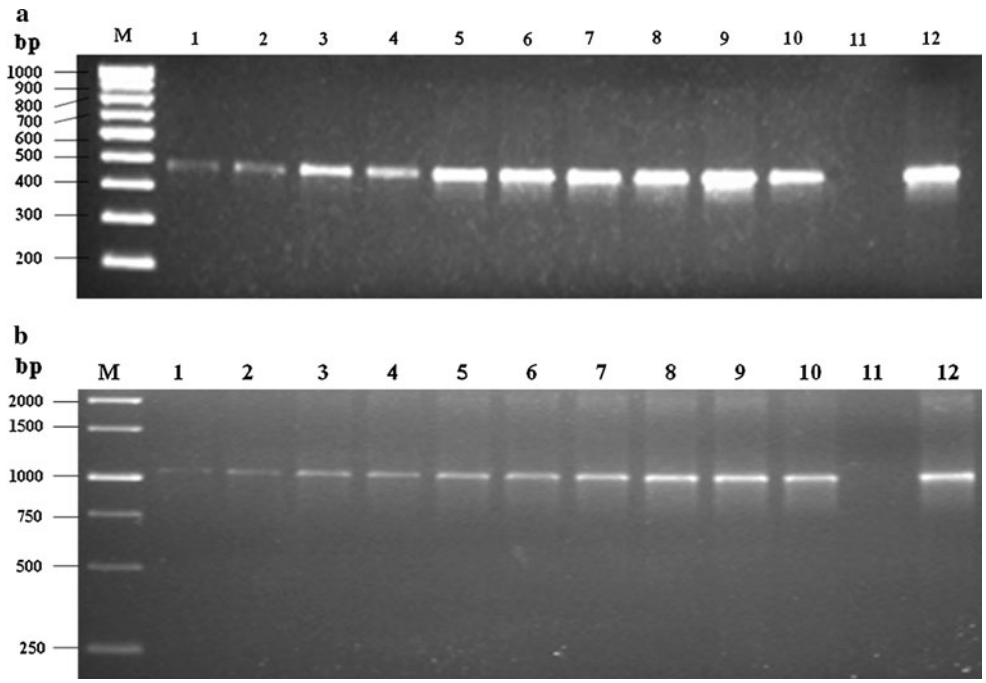

Fig. 3 **a**. PCR amplification of DNA from ICMV-*replicase* gene fragment from individual *B. tabac*i using specific primer pair. The cassava-reared population was given different AAPs on SLCMV-infected cassava plants: *Lanes 1–10* 1, 2, 3, 4, 5, 6, 10, 24, 48 and 72 h AAP, *lane 11* non-viruliferous *B. tabaci* reared on eggplant (negative control), *lane 12* Plasmid DNA contained ICMV *replicase* gene (positive control) and *lane M* 100 bp marker. **b**. PCR amplification of DNA from SLCMV-*replicase* gene fragment from individual B. tabaci using specific primer pair. The cassava-reared population was given different AAPs on SLCMV-infected cassava plants: *Lanes 1–10* 1, 2, 3, 4, 5, 6, 10, 24, 48 and 72 h AAP, *lane 11* non-viruliferous *B. tabaci* reared on eggplant (negative control), *lane 12* Plasmid DNA contained SLCMV *replicase* gene (positive control) and *lane M* 1 Kb marker

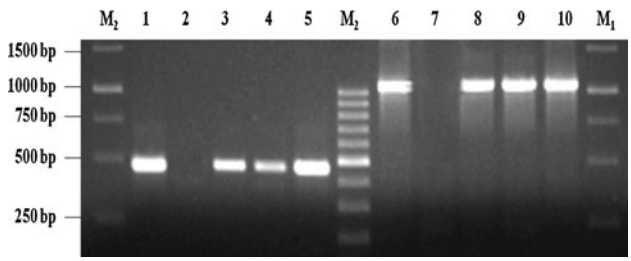

Fig. 4 Colony PCR amplification of ICMV and SLCMV *replicase* gene from individual transformed *E. coli* colonies using specific primer pairs. *Lanes 1* and *6* possitive control, *lanes 2* and *7* negative control, *lanes 3–5* ICMV *replicase* gene transformed *E. coli* colonies, *lanes 8–10* SLCMV *replicase* gene transformed *E. coli* colonies, M_1 1 Kb marker and M_2 100 bp marker

The virus concentration decreases acropetally. Besides, mitosis in the meristem cells competes with virus multiplication, as it occurs rapidly in a dividing callus. Hence the meristem explants of the variety H226 collected from the greenhouse was observed 21 days after inoculation. Among the different MCM series with different growth regulator combination tested, MS basal medium containing 0.1 mg/l of BAP and 0.02 mg/l of GA3 (MCM2) was associated with the greatest meristem establishment in culture medium. A report given by Liu [27] and Nair et al. [30] support this statement. According to them, for meristem culture, BAP was the best growth regulator for morphogenesis, followed by Zeatin and Kinetin. As Nair et al. [30] observed in a previous report concerning the effects of

different levels of the cytokinin BA, with NAA in the presence of GA_3 on cassava meristem-tip regeneration, root development was dependent on cytokinin concentration. The established meristem grew as a single shoot and elongate well with well developed root system was observed in the MCM15 (1.0 mg/l of BAP and 0.5 mg/l GA_3). This result is in accordance with the report by Acedo [4], who stated that MS basal medium supplemented with the combination of 0.25 mg/l GA3, 0.1 mg/l BAP and 0.2 mg/l NAA was the most effective medium for meristem growth and development of Golden yellow cassava variety after about 3 weeks of incubation. The overall regeneration rate of meristem tips in previous studies approached, 40% by Kaiser and Teernba [19], but recent work of this study suggests that 93.11%. Of more interest in these experiments was the total success in transplanting the above regenerants from media to soil. Mortality of transplants had previously been thought to be of little importance in these procedures (e.g. only 2–5% regenerants died when transferred in one report by Kartha and Gamborg [20], but 100% survival on transplantation do occur (results not shown).

To confirm the absence/presence of geminivirus in meristem derived cassava plants of variety H226, DNA was extracted from the above mentioned plants and PCR was carried out using Deng's degenerate primer, along with CMD infected plant as control. When agarose gel electrophoresis of the PCR product was done, an amplified fragment of DNA about 560 bp was obtained. This was obtained only from the DNA isolates from CMD infected H226 cassava plants. While there was no amplification of 560 bp fragment form the DNA of meristem derived virus free cassava plants of these two varieties. This result is in accordance with the report by Manickam et al. [28], who used PCR to detect geminiviruses in dicot weeds and obtained 560 bp of PCR product from *Croton sparsiflorus* and *Eclipta alba* exhibiting yellow vein symptoms.

B. tabaci has been recognized as the vector of cassava mosaic geminiviruses (CMG) for more than a century. More recently, it has also been shown to transmit cassava brown streak virus (CBSV), which causes cassava brown streak disease (CBSD). Super-abundant populations of *B. tabaci* and the recombinant CMG, SLCMV and ICMV-India, have been associated with the expansion of southern states of Tamil Nadu and Sri Lanka of the pandemic of severe CMD. Virus transmission studies using *B. tabaci* showed that 80.5% of transmission efficiency from cassava to cassava during the observation up to 3 months period. Typical CMD symptoms of foliar chlorotic mosaic or mottle, distortion of leaves developed within 22 days. Similar study was also carried out by Chant [10] in earlier. The ACMV vectors, *B. tabaci* biotypes in cassava have been reported from all growing areas of Africa [2, 7, 8, 25] and India [26]. The present study has demonstrated that *B. tabaci* reared on cassava. PCR analysis confirmed the presence of virus acquisition from cassava following 1–72-h AAPs. Similar observations and confirmation were made in India on ICMV [6] and Africa on transmission of ACMV [9, 16, 22] by *B. tabaci*. Legg et al. [25] has also reported the existence of specific *B. tabaci* genotypes in Uganda (Ug1 and Ug2), of which Ug2 is responsible for the spread of the African cassava mosaic epidemic in that region. Legg and Ogwal [24] reported that the incidence of African cassava mosaic disease (ACMD) was higher in north region of Uganda than south due to the presence of higher number of whitefly vectors. Earlier, several workers [2, 5–7, 9, 22, 26]) had reported host specialization of *B. tabaci*. In India, most of the cassava cultivation occurs in the southern part of the country, especially in Kerala and Tamil Nadu. In Salem (Tamil Nadu, India), Cassava is grown throughout the year and hence can serve as permanent host for cassava-adapted *B. tabaci* reproduction. The presence of a permanent host may have triggered the *B. tabaci* adaptation to cassava and the acquired ability to transmit SLCMV and ICMV. The collective results of the present study indicate that SLCMV and ICMV were transmitted by *B. tabaci*. Similarly, in India, ICMV was transmitted by specific Indian cassava biotype of *B. tabaci* occurs which is limited to colonization on cassava [5, 33]. These results and a recent report of several cassava geminivirus isolates from Tamil Nadu, India suggest the possibility of an outbreak of *B. tabaci*/cassava mosaic virus disease in India as has been reported in Uganda.

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