DEVELOPMENT OF INFECTIOUS CLONES OF CASSAVA MOSAIC VIRUS AND THEIR VALIDATION

by

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(2013-09-106)

THESIS

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KERALA, INDIA

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DECLARATION

I, hereby declare that the thesis entitled **"DEVELOPMENT OF INFECTIOUS CLONES OF CASSAVA MOSAIC VIRUS AND THEIR VALIDATION"** is a bonafide record of research done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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DEDICATED TO MY DEAR

PARENTS AND FAMILY

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LIST OF ABBREVIATIONS

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λ	lambda
μ	micron(s)
°C	degree centigrade
μg	micro gram
μl	micro litre
μM	micro molar
bp	base pairs
DNA	deoxyribonucleic acid
kb	kilo base pair
M	molar
mM	milli molar
ng	nano gram
mg	milli gram
nm	nano meter
OD	Optical Density
min	minute
hr	hour
ml	milli litre
cm	centimetre
Amp	ampicillin
Spec	spectinomycin
Strep	sptreptomycin
RNase	Ribonuclease
uv	ultraviolet
Tris	Tris(hydroxymethyl)aminomethane
v/v	volume/volume
w/v	weight/volume
dNTP	deoxy nucleotide triphosphate

pmol	pico mole
pg	pico gram
%	percentage
g	gram
N	Normality
l	Litre
CTAB	Cetylethylammonium bromide
sec	second
U	enzyme unit
SSC	Saline sodium citrate
ha	hectares
$	dollar
rpm	revolutions per minute
TE	Tris EDTA
LB	Luria Broth
LA	Luria Agar
$CaCl_2$	Calcium chloride
YEB	Yeast Extract Beef
3'	Three prime
5'	Five prime
BLAST	Basic Local Alignment Search Tool
E. coli	*Escherichia coli*
FAOSTAT	Food and Agriculture Organization Statistical Database
NCBI	National Centre for Biological Information
ORF	Open Reading Frames
PCR	Polymerase Chain Reaction
RCA	Rolling circle amplification
RCR	Rolling Circle Replication
RNAi	RNA interference
V	Volt
LiCl	Lithium chloride

SDS	Sodium dodecyl sulphate
DIG	Digoxigenin
A_{600}	Absorbance at 600 nanometer

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INTRODUCTION

1. INTRODUCTION

Tuber crops, especially cassava, sweet potato, amorphophallus and yams still continue to be major crops contributing significantly to human and animal food apart from finding use in various industries. These crops are adapted to broad agro-ecological conditions and yield reasonably well even under marginal environments.

Cassava (*Manihot esculenta* Crantz, Family: Euphorbiaceae) is one of the important tuber crops grown in the tropics and can be grown in a wide range of climatic conditions. This crop has high storage capacity and thus ensures food security even during adverse climatic conditions (Lebot *et al.*, 2010). Cassava starch is being used for its industrial applications like, bio ethanol production, processing in paper industry, and as pellets for animal feed (Blagbrough, 2010). In India, cassava is grown in an area of 2.04 lakh ha with an annual production of 45.54 lakh tonnes (FAOSTAT, 2016). While in Kerala, it is grown in an area of 75,493 ha with an annual production of 29.43 lakh tonnes (Analytical study on agriculture in Kerala, 2016).

Among the various diseases that affects the crop, cassava mosaic disease (CMD) is the major threat in cassava production and leads to yield loss of about 88 percent in susceptible varieties and 50 percent in field tolerant varieties (Malathi *et al.,* 1985; Obonyo, 2007). CMD is caused by cassava mosaic geminiviruses (CMGs) belonging to the Geminiviridae family and *Begomovirus* genera. These viruses are transmitted by whitefly *Bemisia tabaci* and also through vegetative propagation (Legg and Fauquet, 2004; Legg *et al.*, 2006).

CMD causing begomoviruses are bipartite, that is, they have two circular single stranded DNA components, DNA-A and DNA-B. The DNA-A contains genes that codes for viral encapsulation, transcription enhancement and replication whereas DNA-B contains genes that codes for movement of viral DNA within and between the host cell (Kheyr-Pour *et al.*, 1991; Fauquet *et al.*,

2008). These viruses replicate in the nuclei of infected cells through rolling circle replication (RCR) mechanism (Hanley-Bowdoin *et al.*, 1991).

Only after 1900s, research on CMD became prominent. That was due to growing importance of cassava in food industries and widespread occurrence of CMD infection during famine. The disease is characterized by a yellow-green mosaic symptom on the leaves, leaf distortion, chlorosis, stunted growth and reduction in the size of the root.

Across the world, CMD is caused by twelve different cassava mosaic viruses (Legg *et al.,* 2015). In India only two of them are prominent, namely, *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV). They cause yield loss of up to 80% in susceptible varieties and 20-30% in improved ones (Malathi *et al.*, 1985; Hegde *et al.,* 2010). It has been found that the recombinogenic potency of SLCMV is higher than that of ICMV. CMD has caused havoc in Africa and Asia covering an area of 2.6 million square kilometres and causing an estimated loss of US$ 1.9 to 2.7 billion per annum (Patil *et al.,* 2009). This CMD infection is usually detected by diagnostic methods like Enzyme Linked Sorbent Assay (ELISA) and Polymerase Chain Reaction.

The objectives of my work were

- Cloning and characterization of SLCMV/ICMV infected field samples.
- Construction of infectious clones of SLCMV/ICMV.
- Agroinoculation of *Nicotiana benthamiana* with the partial dimers constructed to check the infectiousness of the viral clones.

Usually, screening of cassava plants for CMD resistance is very difficult and so advancement in cassava breeding for CMD resistance is lagging behind. Therefore, developing efficient infectious clones of cassava mosaic virus and their subsequent inoculation technique would provide a major advancement to the resistance development in cassava. Findings from the current study will also be useful for assays evaluating the resistance of transgenic cassava plants to geminiviruses.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 CASSAVA (*Manihot esculenta* Crantz)

Cassava (*Manihot esculenta* Crantz) which belongs to the Euphorbiaceae family is a vegetatively propagated woody shrub with edible tubers that can be grown in adverse climatic conditions. It originated in South America and now is the staple food for millions of people in developing countries across the world. Since it is widely cultivated in the tropics, it is called as "bread of the tropics".

Cassava plants reach up to 4 meters in height at maturity. The tuberous roots may be harvested from 1 to 4 years after planting. These roots have high starch content and make up to 30- 40%. These plants produce fertile seeds, but usually stem cuttings are used for propagation.

Cassava was introduced to India during the 17th century as a food crop but the potential of this crop was recognized only when the Second World War started and got its name as 'famine saver'. In India, cassava is grown in an area of 2.04 lakh ha with an annual production of 45.54 lakh tonnes (FAO, 2016). It is mostly grown in Kerala, Tamil Nadu, Andhra Pradesh and also in some North Eastern states (Anitha *et al.,* 2011). In Kerala, it is grown in an area of 75,493 ha with an annual production of 29.43 lakh tonnes (Directorate of agriculture-Kerala, 2016). While in Tamil Nadu, it is grown in an area of 90,510 ha with an annual production of 35.12 lakh tonnes (Dept. of agriculture- India, 2017).

Cassava is an important source of Vitamin B complex. This crop has high storage capacity and thus ensures food security even during adverse climatic conditions (Labot *et al.*, 2010). Cassava starch is being used for its industrial applications like, bio ethanol production, processing in paper industry, and as thickeners in the food industry (Blagbrough, 2010). It is also a source of income for thousands of poor farmers in Africa and Asia. Due to its high tolerance towards abiotic stress and other unfavourable conditions, cassava has become a successful crop in the tropics when compared to other crops such as wheat, rice and maize, for farmers with very limited resources (Blagbrough, 2010). These

tubers can be left behind in the ground for longer periods of time, thus protecting farmers from famine conditions.

2.2 GEMINIVIRUSES

Geminiviruses (Family:*Geminiviridae*) are circular single-stranded DNA (ssDNA) plant pathogens, that causes damage to many crop plants and are transmitted by insect vectors. They replicate via double stranded DNA (dsDNA) intermediates in the infected cells. They depend on their host plant for enzymes needed in replication and transcription of viral genome.

On the basis of genome organisation, host range and nature of the insect vector, they are classified into nine genera, *Begomovirus, Becurtovirus, Grablovirus, Eragrovirus, Turncurtovirus, Capulavirus, Mastrevirus, Curtovirus and Topocuvirus* (Fig. 1). Viruses in the *Begomovirus* genera have bipartite or monopartite genome, while those belonging to other genera have monopartite genome (Brown *et al.*, 2012; Varsani *et al.,* 2017). Viruses belonging to *Begomovirus* genera, cause huge loss in crop production and they are transmitted by whitefly that belongs to the species *Bemisia tabaci* (Sanderfoot and Lazarowitz, 1996).

Bipartite genome is designated as DNA-A and DNA-B, both having 2.7 kb size (Kushawaha *et al.*, 2015). The DNA-A contains genes that codes for viral encapsulation and replication whereas DNA-B contains genes that codes for movement of viral DNA within and between the host cell (Kheyr-Pour *et al.*, 1991; Fauquet *et al.*, 2008). Even though they are similar in size, sequences are different except for a common region (CR) within the intergenic region. Within the CR region, there is a highly conserved sequence called nonanucleotide sequence (TAATATTAC) which constitutes the origin of replication (ori).

The genes present in DNA-A are *AC1* [(Rep) Replication associated protein], *AC3* [(REn) replication enhancer protein], *AC2* [(TrAP) Transcriptional activator protein], *AV1* [(CP) Coat protein], *AC4* (AC4 protein). While the DNA-B encodes two proteins, BC1 [(MP) Movement protein] and BV1 [(NSP) Nuclear

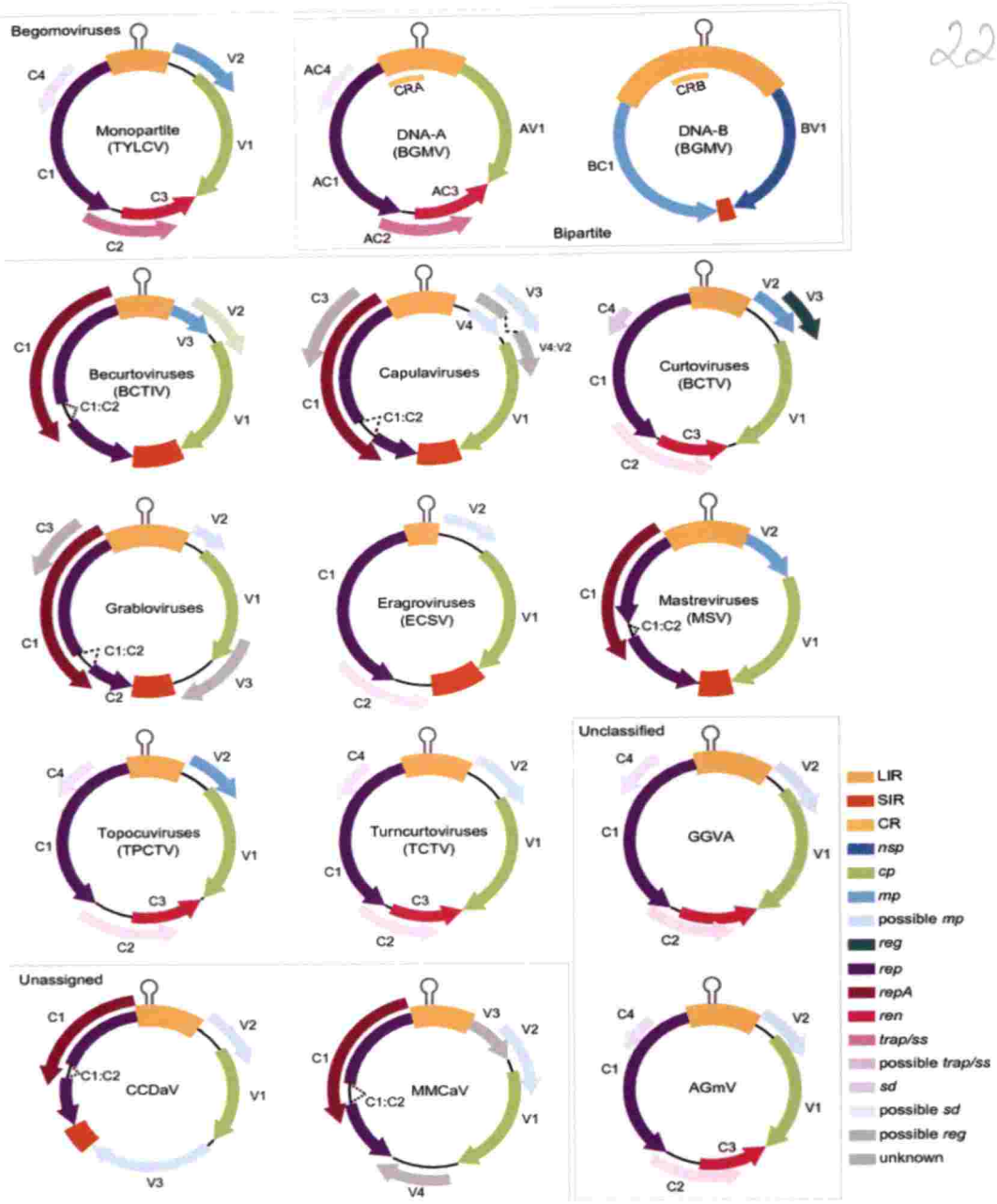

Fig.1 Geminivirus classification (Vasrsani *et al.*, 2017)

shuttle protein], involved in viral movement within the plant (Stanley *et al.*, 2004) (Fig. 2). AC2 and AC4 have also been found to act as suppressors of RNA interference (RNAi), a defence response of plants against viruses (Vanitharani *et al.*, 2004). DNA A part can replicate on its own but requires the DNA B encoded BV1 for nuclear localisation process and BC1 for intrinsic infection.

Geminiviruses are highly recombinogenic, as shown by their tendency for rearrangement of their genomic DNA and transfer of sequences between genomic units (Etessami *et al.,* 1989). Begomovirus diversity has been mainly due to interspecific recombination events (Padidam *et al.,* 1999).

2.2.1 Life cycle

Geminiviruses replicate in the nuclei of infected cells through rolling circle replication (RCR) mechanism (Hanley-Bowdoin *et al.*, 2013). Once the single stranded viral DNA is released from virions, it is copied to generate double-stranded DNA and thus infection begins in the plant. RNA polymerase II of the host transcribes the dsDNA that attaches with nucleosomes, allowing synthesis of replication initiator protein (Rep). Rolling-circle replication is initiated by Rep and introduces a nick in the conserved nonanucleotide sequence (TAATATTAC) (Fontes *et al.* 1994; Stanley 1995; Stenger et al., 1991). This in turn generates a free 3' hydroxyl end which extends in the 5'-3' direction. The concatemeric DNA forms thus synthesised are cleaved by Rep and ligated afterwards for the production of ss circular viral DNA (Gutierrez, 2000; Yadava *et al.*, 2010).

Rep represses its own transcription, leading to activation of transcriptional activator protein (TrAP) expression, which in turn activates coat protein (CP) and nuclear shuttle protein (NSP) expression. Circular viral DNA can then be encapsulated by CP into virions, which are available for whitefly procurement. NSP binds to viral DNA and moves it across the nuclear envelope, where movement protein (MP) helps in crossing the plasmodesmata.

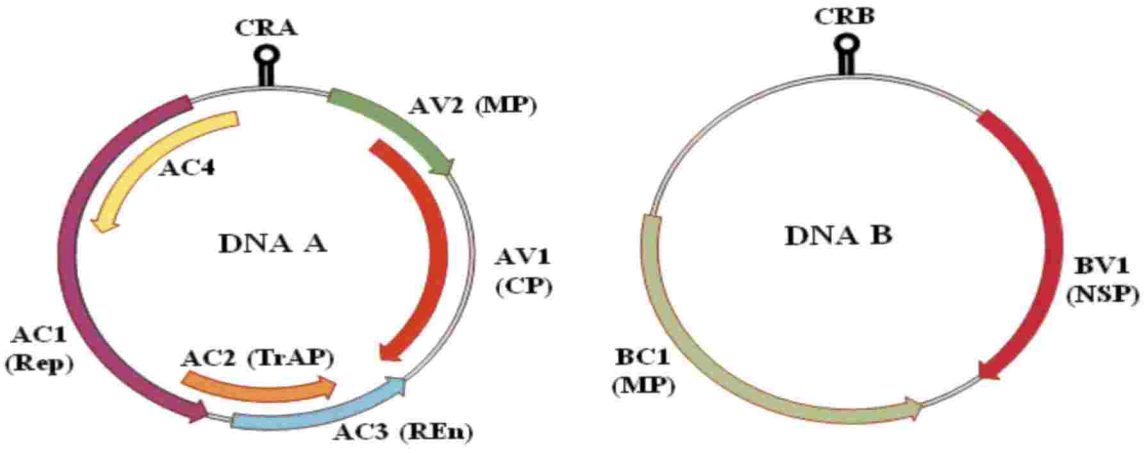

Fig. 2. Typical genome organization of cassava-infecting begomoviruses DNA A and DNA B components. CRA (common region A); CRB (common region B); CP (coat protein); MP (movement protein); Rep (replication associated protein); TrAP, (transcriptional activator protein); REn, (replication enhancer protein) (Adapted from Xie *et al.*, 2010).

Fig. 3: Common symptoms of cassava mosaic virus in cassava

2.3 CASSAVA MOSAIC DISEASE

Cassava mosaic disease (CMD) is one of the major plant viral disease that significantly reduces the cassava productivity and so is a major biotic restraint in cassava production (Legg *et al.*, 2006). It is caused by cassava mosaic geminiviruses (Family: *Geminiviridae*; Genus: *Begomovirus*) and is transmitted by whitefly vector, *Bemisia tabaci*. Whitefly population is able to transmit the viruses because of the specific adaptation of its coat protein (Harrison and Robinson, 1999).

There are twelve species of cassava mosaic begomoviruses namely: -

1. *Indian cassava mosaic virus* (ICMV)
2. *Sri Lankan cassava mosaic virus* (SLCMV)
3. *African cassava mosaic virus* (ACMV)
4. *South African cassava mosaic virus* (SACMV),
5. *East African cassava mosaic virus* (EACMV)
6. *East African cassava mosaic Cameroon virus* (EACMCV)
7. *East African cassava mosaic virus* – Uganda (EACMV-UG)
8. *East African cassava mosaic Zanzibar virus* (EACMZV)
9. *East African cassava mosaic Malawi virus* (EACMMV)
10. *East African cassava mosaic Kenya virus* (EACMKV)
11. *African cassava mosaic Burkina Faso virus* (ACMBFV)
12. *Cassava mosaic Madagascar virus* (CMMGV)

(Legg *et al.*, 2015)

Among these viruses, *Indian cassava mosaic virus* (ICMV) was the first cassava mosaic gemimivirus found out from South Asia (Hong *et al.*, 1993), followed by *Sri Lankan cassava mosaic virus* (SLCMV) (Saunders *et al.*, 2002, Dutt *et al.*, 2005).

Cassava mosaic disease has caused havoc in Africa and Asia covering an area of 2.6 million square kilometres and causing an estimated loss of US$ 1.9 to 2.7 billion per annum (Patil *et al.*, 2009).

Cassava mosaic disease could be eliminated by the use of meristem culture to produce virus free planting material in cassava (Deepthi and Makeshkumar, 2016). The resulting virus free plant material showed best performance in filed with respect to yield when compared with conventional planting material.

2.3.1 Regulation

Regulation of transcription in begomoviruses has been studied widely in both protoplast systems and transgenic plants (Frey *et al.*, 2001; Shivaprasad *et al.*, 2005). Rolling circle replication is the mode of replication seen in begomoviruses. This in turn produces double stranded DNA which acts as templates for transcription and the transcriptional process happens in a bidirectional manner with the help of bidirectional promoter located within the intergenic region. *AC2* is a viral transcription factor that trans-activates the late viral genes *AV1* and *BV1* (Sunter and Bisaro, 1992).

2.3.2 Cassava mosaic virus in the Indian subcontinent

CMD was first reported in India by Abraham (1956). After that it was reported by Austin in Sri Lanka (1986). Among different cassava growing states, overall occurrence of CMD is highest in Tamil Nadu (30 %) and Kerala (23 %) (Calvert and Thresh, 2002).

The existence of SLCMV in Southern India was reported through the differential PCR studies and a distribution map for ICMV and SLCMV was developed (Patil and Dasgupta, 2005; Patil *et al.*, 2005). Afterwards, various ICMV and SLCMV isolates from southern India were cloned and their infectivity and biodiversity were studied (Dutt *et al.*, 2005; Patil *et al.*, 2007; Rothenstein *et al.*, 2006).

The result of a survey conducted by Anitha *et al.* in 2011 indicated that cassava mosaic disease occurred throughout the state of Kerala at low to high incidences (44-96%) and SLCMV is widespread in Kerala compared to ICMV.

2.3.3 Plant host range

ACMV is usually restricted to Solanaceae family and within which, it is more easily transmitted to plants of genera like *Nicotiana* and *Datura* (Bock and Woods, 1983). *N. tabacum* and *N. Glutinosa* are the *Nicotiana* species readily infected by ICMV and SLCMV. SLCMV is highly virulent and has a wide host range extending to *Arabidopsis* (Mittal *et al.*, 2008) and even to *Ageratum conyzoides* (Saunders *et al.*, 2002). Infection due to this virus will lead to mosaic patterns in the leaves of the host plant.

2.3.4 Transmission

One of the major causes for the spread of cassava mosaic disease is the extensive use of infected planting material. Another cause of spread is the whitefly vector, *Bemisia tabaci*. It has been found that CMD is not spread through seeds (Mathew, 1989; Makeshkumar *et al.*, 2005), but it can be easily transmitted through grafting (Edison *et al.*, 2004). It has been reported that cassava mosaic viruses could be transmitted by mechanical inoculation from cassava to various species like *Datura stramonium, Nicotiana* sp., *Manihot glaziovii*, *Petunia hybrida* and *Nicandra phylosalodes* (Malathi *et al.*, 1983; Mathew *et al.*, 1993; Anitha *et al.*, 2008).

2.3.4 Symptomatology

It was in 1938, Storey and Nicholas for the first time described the symptoms of CMD. Cassava mosaic disease is seen as discrete leaf mosaic patterns in various parts of the leaf that occurs even at the initial stages of plant development. Pale green or yellow chlorotic mosaic on leaves, followed by distortion and crumbling are the usual symptoms of cassava mosaic virus infection. These chlorotic zones are normally well differentiated and differ in size from that of a whole leaflet to small spots. Leaflets may show a uniform mosaic pattern or the mosaic pattern is localised to a few areas which are often at the bases (Fig. 3).

Symptoms differ from shoot to shoot, leaf to leaf and plant to plant, even of the same virus strain and plant variety in the same locality. Differences in symptoms may be due to variation in the sensitivity of the host genome, virus

stain, age of plant, and environmental factors such as soil moisture, soil fertility, radiation and particularly temperature.

Several leaves situated in between affected ones may appear as normal and give signs of recovery. This behaviour is influenced by the suitable temperature and host-plant resistance. However, symptoms may reappear on recovered plants when environmental conditions again favour expression of symptoms (Gibson and Otim-Nape, 1997). Symptoms tend to recur on the auxiliary growth after the shoot tips are decanted. De-topping is sometimes used to augment expression in screening clones for resistance (Jennings, 1960). In case of resistant plants, they may recover from the infection and produce symptomless leaves during later stages of life and especially during hot weather conditions (Malathi *et al.*, 1985; Edison *et al.,* 2007; Anitha *et al.*, 2011; Makeshkumar *et al.,* 2015).

However, CMD symptoms may be confused with the symptoms seen when leaf is damaged due to cassava mealybug (*Phenacoccus manihoti*), cassava green mite (CGM) (*Mononychellus tanajoa*), cassava bacterial blight (*Xanthomonas campestris* pv. *manihotis*), drought and mineral deficiencies (Sseruwagi *et al.*, 2004).

2.4 DETECTION AND DIAGNOSIS OF CASSAVA MOSAIC DISEASE

Cassava mosaic disease could be identified by visual symptoms and subsequent scoring of the symptoms. But these symptoms are highly variable and are similar to the incidence of dryness or nutrient deficiency seen in plants. So, such scoring of visual symptoms has certain limitations and is not widely used.

One of the sensitive and simple methods for diagnosis and quantification of virus titer in the plant is Enzyme-Linked Immunosorbant Assay (ELISA) (Clark and Adams, 1977). Givord *et al.* (1994) first reported that ELISA has been used for the diagnosis of several geminiviruses. One of the limitations of ELISA is that, it is not possible to differentiate between different cassava mosaic viruses present in mixed virus infections (Thottappilly *et al.*, 2003).

In India, different cassava mosaic viruses in plants were detected using Polymerase Chain reaction (PCR) (Makeshkumar *et al* 2005; Rajinimala *et al.*, 2007; Khan *et al.*, 2011; Shery *et al.*, 2016). At first, in order to detect any geminivirus, conserved regions of geminivirus were used (Wyatt and Brown, 1996). Later on, specific primers were designed from full length sequences of cassava mosaic viruses and used for the detection of ACMV, ICMV and SLCMV in CMD infected plants (Makeshkumar *et al.*, 2001; Dutt *et al.,* 2005; Patil *et al.*, 2005). Multiplex PCR can be used for the detection and differentiation of EACMV and ACMV in Africa as well as SLCMV and ICMV in India (Dutt *et al.*, 2005; Patil *et al.*, 2005).

2.5 WHOLE GENOME AMPLIFICATION

RCA is a simple amplification method used to obtain the whole genome of an organism (Fujii *et al.*, 2004). It is an isothermal method and does not require any specific primer for amplification. This method is very suitable for the amplification of small circular DNA because of its proof-reading activity, strand displacement function and production of long synthesis products (Johne *et al.*, 2009). The DNA polymerase produced from bacteriophage *phi29* is being used as enzyme in this reaction. The products of this method are linear DNA duplexes consisting of tandem repeats of the circular DNA sequence called 'concatemers'. The average synthesis rate in RCA is 1500 nucleotides per minute and thus 900 kb is produced within 10 hrs. RCA was used for the cloning of single-stranded circular DNA genome of a geminivirus in 2004 (Inoue-Nagata *et al.*, 2004). The ideal RCA products can be identified by digestion using appropriate restriction enzymes.

Benefits of low cost, easy of handling, ability to detect all infecting circular parts in a single step without any prior sequencing knowledge, gave wide recognition for this technique all over the world for the detection of CMBs (Kushwaha *et al.*, 2010).

In this reaction, random hexamers (NNNNNN) are hybridized with circular DNA, and the resulting double-strand segments function as primers in the polymerization reaction carried out by *phi29* DNA polymerase, a unique enzyme with very high strand-displacement activity. As the 'front' of the extending complementary strand of the plasmid encounters double-stranded portions of DNA, the advancing new strand displaces the old one from the template. This extension process covers the entire length of the circular DNA multiple times, resulting in the formation of repeated sequences of the template, called concatemers. The hexamers also hybridize with these concatemers, which become templates in their own right. This extension, however, proceeds only until the terminus of the linear concatemer is reached. The result is the formation of various lengths of double-stranded DNA consisting of repeats of the template sequence. The mechanism is shown in Fig. 4.

2.6 METHODS TO INTRODUCE GEMINIVIRUSES

The method of inoculating geminiviruses depends on the virus and host combination: (a) direct transmission or from an infected plant through an insect vector (b) mechanical rubbing (c) the engineering and cloning of partial dimers into *Agrobacterium tumefaciens* vector for agroincoulation and (d) biolistic delivery. The detailed list of geminiviruses for which infectious clones were developed, plants where it was demonstrated, different mode of delivery of those infectious clones are shown in Table 1.

2.6.1 *Agrobacterium* mediated gene transfer

*A. tumefaciens*is a gram-negative soil bacterium, belonging to the *Rhizobiaceae* family. It is a soil phytopathogen that naturally infects the wound sites in dicotyledonous plants leading the formation of crown gall tumors. It was in 1907, Townsend and Smith first noted that crown gall tumors are caused by *A. tumefaciens.*

A. tumefaciens is used by many scientists in molecular studies to introduce genetic material (DNA) into plants. During infection by these bacteria, a piece of

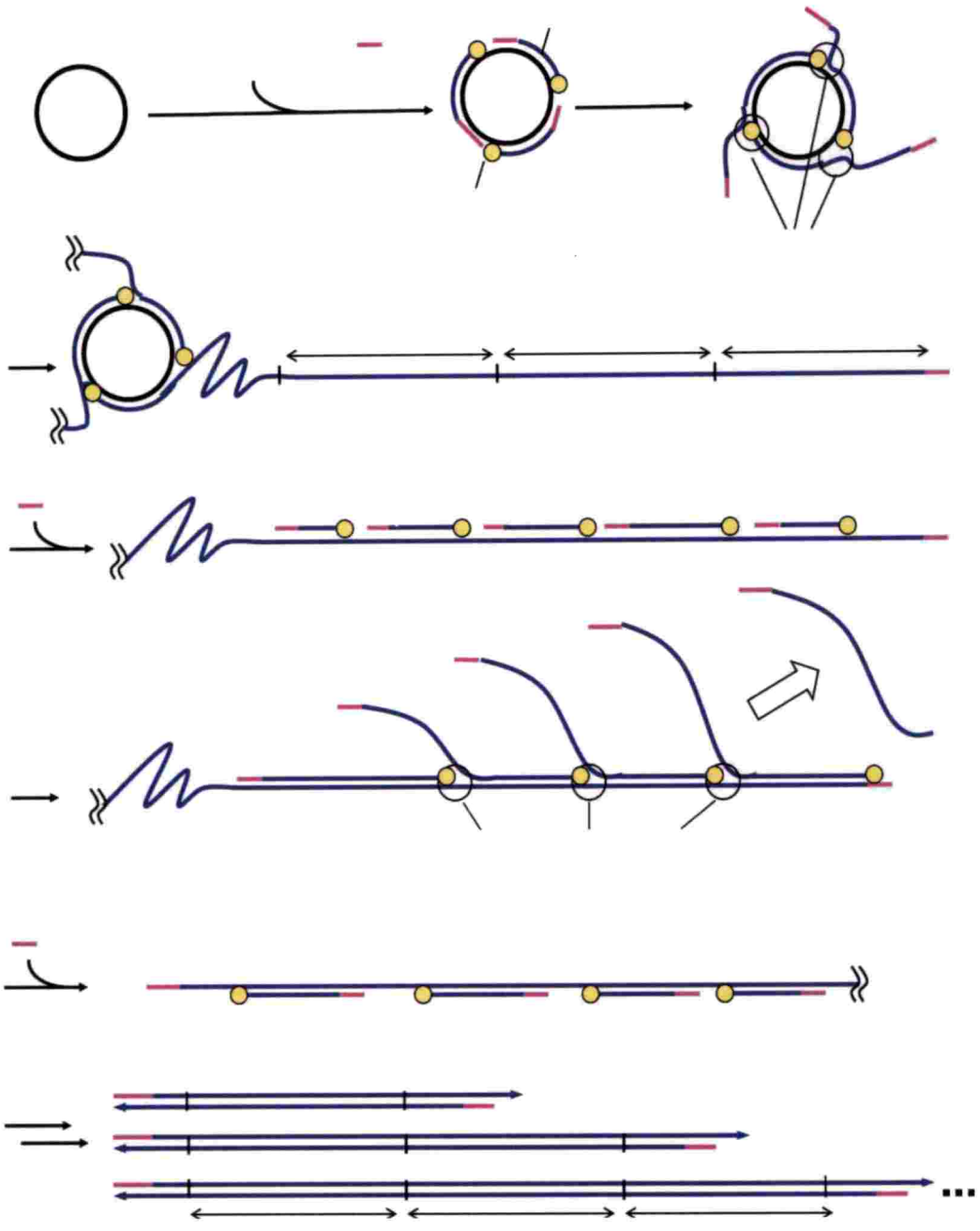

Fig. 4: Mechanism of rolling circle amplification (Fujii *et al.*, 2006)

Table 1. The detailed list of geminiviruses for which infectious clones were developed, plants where it was demonstrated, different mode of delivery of those infectious clones.

S.No.	Virus name	Test plants	Mode of delivery	References
1	Sri lankan cassava mosaic virus	*N. benthamiana,* Arabidopsis, cassava	Agroinoculation, biolistic (cassava)	Mittal *et al., 2008*
2	Indian cassava mosaic virus	*N. benthaminana,* cassava	Agroinoculation, biolistic (cassava)	Rothenstein *et al.,* 2005
3	Cassava latent virus	*N. benthamiana*	Agroinoculation	Stanley *et al.,*1983
4	African cassava mosaic virus	Cassava	Biolistic	Briddon et al., 1998
5	East African cassava mosaic virus	Cassava	Biolistic	Ariyo *et al.,* 2006
6	East African cassava mosaic virus-Uganda	Cassava	Biolistic	Ariyo *et al.,* 2006
7	Beet curly top virus	*Beta vulgaris, N. benthamiana*	Agroinoculation	Stanley *et al.,* 1986
8	Sweet potato leaf curl virus	*Ipomoea betatas*	Agroinoculation(vacuum infilteration)	Bi and Zhang 2014
9	Pepper huasteco virus	Pepper	Biolistic	Bonilla-Ramirez *et al.,* 1997
10	Panicum streak virus	*Zea mays*	Agroinoculation	Briddon *et al.,* 1992
11	Potato yellow mosaic virus	*Tomato, N. benthamiana*	Agroinoculation	Buragohain *et al.,* 1994

12	Bhendi yellow vein mosaic virus	*N. benthamina*	Agroinoculation	Chandran *et al.*, 2013
13	Maize streak virus	Maize	Agroinoculation	Grimsley *et al.*, 1987
14	Mungbean yellow mosaic virus	Black gram, mung bean, French bean	Agroinoculation	Haq *et al.*, 2011
15	Legume yellow mosaic virus	Soyabean	Agroinoculation	Ilyas *et al.*, 2009
16	Tomato gold mosaic virus	*N. benthamiana*	Agroinoculation	Hamilton *et al.*, 1983
17	Tomato yellow leaf curl virus	Datura, tomato	Biolistic	Lapidot *et al.*, 2007
18	Tomato leaf curl Palampur virus	*N. benthamiana*	Agroinoculation	Malik *et al.*, 2011
19	Vernonia yellow vein virus	*Vernonia cinerea*	Agroinoculation	Packialakshmi and Usha 2011
20	Bean golden mosaic virus	*Phaseolus vulgaris*	Agroinoculation	Morinaga *et al.*, 1983
21	Digitaria streak virus	*Digitaria sanguinalis, Zea mays, Avena zativa*	Agroinoculation	Donson, 1988
22	Ageratum yellow vein Taiwan virus	*N. benthamiana*	Agroinoculation	Wu *et al.*, 2008
23	Chilli leaf curl Palampur virus	*N. banthamiana,* chilli	Agroinoculation	Kumar *et al.*, 2011
24	Ageratum enation virus	*N. benthamiana*	Agroinoculation	Tahir *et al.*, 2015
25	Papaya leaf curl China virus	*N. benthamian, Solanum lycopersicum, Petunia hyrida*	Agroinoculation	Zhang *et al.*, 2010

DNA which is a copy of the T-DNA is transferred from the bacterium to the plant cell. The Ti-plasmid or tumor inducing plasmid is the one which carries the T-DNA. The T-DNA is flanked by 25-bp direct repeats that act as border on either side. This flanking region acts as *cis* elements needed for T-DNA processing. All DNA present in between these borders will be transferred to plant cell. Usually, wild-type T-DNA contains enzymes needed for the production of plant hormones like cytokinin and auxin. These hormones are the ones that induce tumorous phenotype in plants. The T-DNA also encodes enzymes needed for the synthesis of opines.

Several enzymes needed for catabolism are encoded by Ti-plasmid, hence, *Agrobacterium* has progressed to genetically commandeer plant cells and use them to produce compounds that they exclusively can utilize as a carbon/nitrogen source.

After the T-DNA mediated transfer of the partial dimer of a virus into plant cells, a unit-length viral constituent is freed which replicates as a circular, double-stranded replicative form either by replicational-release mechanism or by homologous recombination (Stenger *et al.*, 1991) (Fig. 5).

There are two different ways for the discharge of unit-length geminivirus DNAs. Tandemly repeated copies in the inoculum can give rise to a circular dsDNA by recombination. Alternatively, replication between plus strand origins may produce a ssDNA that can be converted to circular dsDNA.

2.6.2 Agroinoculation

Agroinoculation is a process that uses *Agrobacterium tumefaciens* for transferring infectious clones into the plants. They transfer binary vectors having a tandem repeat of a cloned viral DNA into host cells (Grimsley *et al.*, 1986 and 1987). This in turn spreads fully over the plant and symptoms of infection arise.

Even though these molecules are biologically active when in naked form, it was not sure that, *Agrobacterium* would act as an effective alternative to natural vectors. For the infection to begin, autonomously replicating molecule must be

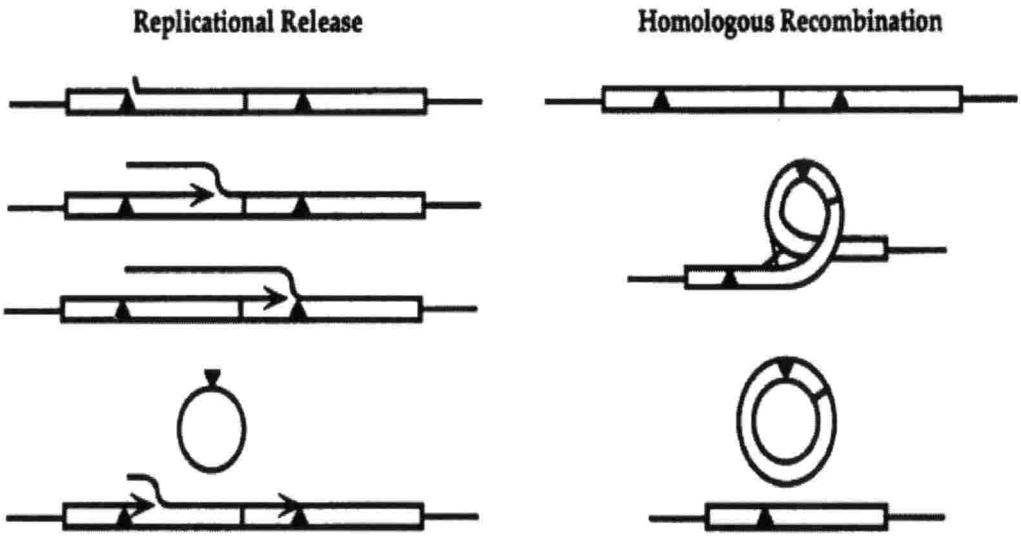

Fig.5: Models for release of unit-length viral DNA from tandem repeats (Stenger *et al.*, 1991)

liberated from the T-DNA in the plant. Agroinfection of maize by MSV showed that cloned MSV isolate was infectious and that the T-DNA transfer was possible to a graminaceous plant (Grimsley *et al.*, 1987). Agroinoculation was first applied to study about *Cauliflower mosaic virus* (CaMV) and *Maize streak virus* (Grimsley *et al.*, 1986 and Grimsley *et al.*, 1987).

Agroinfection produces localised infection in the vascular bundles of plants, near to the area of inoculation and those infected ones gives inoculum for systemic infection in the plant (Hou *et al.*, 1998). The efficiency of agroinfection is dependent on the functions of *vir* genes on the Ti plasmid (Grimsley *et al.*, 1989), type of *Agrobacterium* strain employed (Boulton *et al.*, 1989), and the strength of promoter upstream of the viral replication associated protein. Usually, agroinfection is done by introducing *Agrobacterium* into plants using a needless syringe. This method has been corrected and standardised for several plant species (Wroblewski *et al.*, 2005).

Agroinoculation has been found to be successful in introducing geminiviruses into plant's leaf portion, stem region, and germinating seeds (Czosnek *et al.*, 1993; Kheyr-Pour *et al.*, 1994). In 2001, Biswas and Varma developed two types of agroinoculation such as, sprouted seed and seedling inoculation and were found to be very effective.

Usually, SLCMV is found to be more severe in causing disease in *N. benthamiana* when compared to ICMV. They show symptoms like chlorosis, curling of leaves, stunting. When SLCMV DNA A clone alone was introduced to *N. benthamiana,* upward leaf curl symptom was observed similar to those caused by monopartite begomoviruses (Saunders *et al.,* 2001). Also, symptoms related with SLCMV infection are similar to ACMV infection of their host (Stanley, 1983).

2.6. Advantages of agroinoculation

Agroinoculation is a cheaper method used by researchers to study about the infectivity of different viruses on plants. Inoculation efficiency is also superior in agroinoculation when compared to other methods such as biolistic delivery.

It is also helpful in knowing the plant-virus interactions, to study the replication of viral DNA inside the host, and to assess the resistance of transgenic plants to geminiviruses (Hanley-Bowdoin *et al.*, 1999, 2004; Shepherd *et al.*, 2005).

2.6.4 Factors for selecting binary vector and *Agrobacterium* strain

Viral sequences were not deleted when electroporation was used for transferring the binary plasmid containing viral DNA. Deletion occurred in partial dimers of both DNA A and DNA B that carries the duplication of viral *ori* flanking sequences. The partial dimers in binary vectors like pGA472 and pBin19 having RK2 replicon underwent deletion whereas those in pPZP having pVS1 replicon did not undergo deletion. It was found that *Agrobacterium* strains having C58 chromosome underwent deletion whereas no deletion occurred in strains *Ach5* and *T37*. Thus, it was concluded that chromosomal background of *A. tumefaciens* recipient strains is a very important factor in determining the deletion of viral sequences from partial dimers (Shivaprasad *et al.*, 2006).

Deletion of viral sequences in the partial dimers happens during conjugal transfer as the plasmid DNA is transferred in single stranded form. Usually single stranded DNA is found to be better substrate for recombination than double stranded ones and this recombination in turn leads to deletion (Tinland *et al.*, 1994; Zhao *et al.*, 2003).

With this background and identifying the gaps, the present study has been carried out with an aim of developing efficient clones of cassava mosaic virus in order to screen transgenic cassava plants resistant to CMD.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Development of infectious clones of cassava mosaic virus and their validation" was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI), Sreekariyam, Thiruvananthapuram during 2017-2018. Details regarding the experimental materials and methods used in the study are elaborated in this chapter.

3.1 COLLECTION OF INFECTED LEAF SAMPLES

Cassava mosaic virus infected samples were collected from the fields in ICAR-CTCRI, Thiruvananthapuram. Different leaf samples were classified (Fig. 6) according to the symptom scale of Hahn *et al.* (1980) (Table 2). The photos of the leaf samples were taken, kept in polythene covers and stored at -80°C for further use.

3.2 PLANT DNA ISOLATION

Plant DNA was isolated from the cassava leaf samples by CTAB method (Lodhi *et al.,* 1994). To the CTAB extraction buffer (Appendix I), β-mercaptoethanol was added freshly and made the final concentration as 0.2 % (v/v). Then CTAB buffer was pre-warmed to 60°C for 5 mins. About 100 mg of leaf samples were weighed and ground to fine powder in sterile mortar and pestle using liquid nitrogen. The fine powder was then transferred to a 2 ml centrifuge tube containing 1 ml CTAB extraction buffer. The contents were mixed well by gentle inversion and incubated at 60°C for 30 minutes in waterbath (ROTEK, India). Then tubes were centrifuged at 12,000 rpm for 10 mins at room temperature. The supernatant was transferred to new centrifuge tubes using a sterile pipette tip. To this, 10 µl activated RNase (10mg/ml) was added and incubated for 1 hr at 37°C. Equal volume of chloroform: isoamyl alcohol (Appendix I) was added and mixed well by inversion until the solution turns milky white in colour. Then tubes were centrifuged (Hermle, Table top refrigerated centrifuge) at 14,000 rpm for 10 mins at 4°C. To the upper phase, 0.8 volume of ice cold isopropanol was added and mixed well. The samples were kept at -20°C for overnight incubation in order to precipitate the nucleic acid present. On the next day, samples were centrifuged at 14,000 rpm for

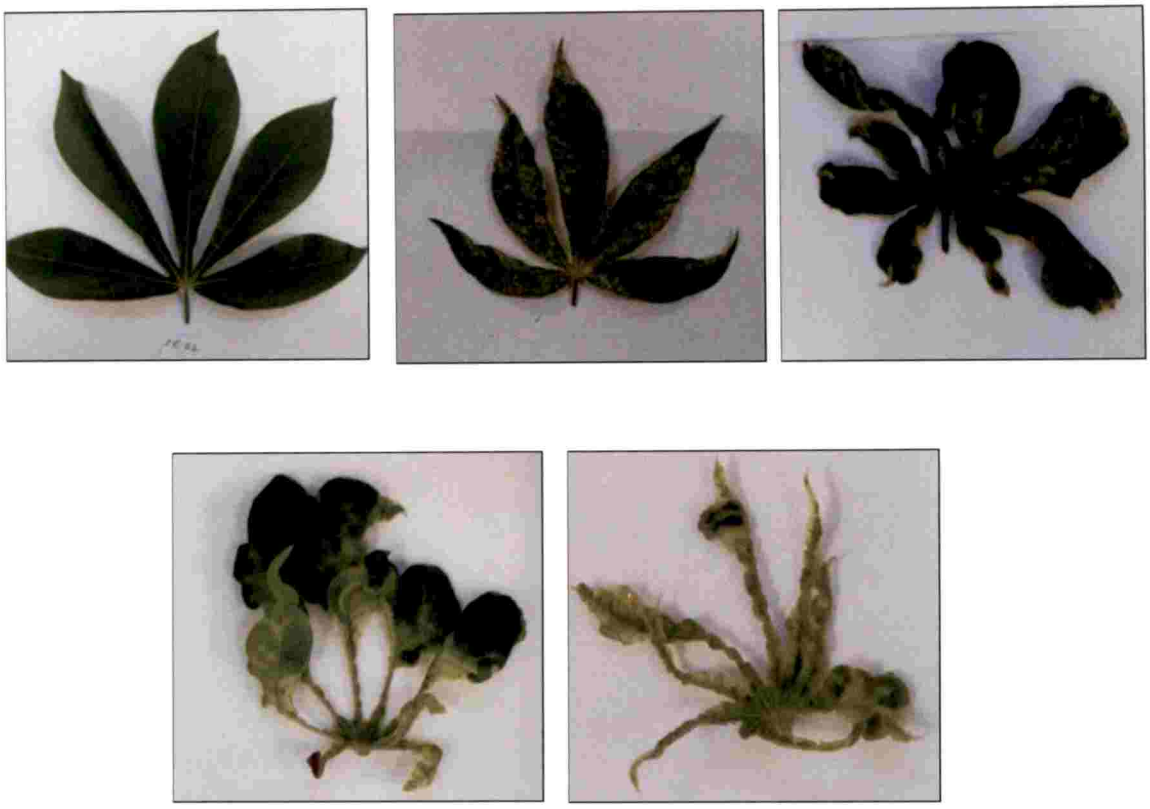

Fig. 6: The cassava mosaic symptom scale of 1-5. 1-Unaffected leaves, no symptoms; 2- Mild chlorosis, mild distortions at bases of most leaves, while the remaining parts of the leaves and leaflets appear green and healthy; 3- Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets; 4- Severe mosaic distortion of two thirds of most leaves and general reduction of leaf size and stunting of shoots; 5- Very severe mosaic symptoms on all leaves, distortion, twisting, misshapen of most leaves accompanied by severe stunting of plants.

Table 2. The cassava mosaic symptom scale of 1-5 (Hahn *et al*, 1980)

Scale	Description
1	Unaffected shoots, no symptoms.
2	Mild chlorosis, mild distortions at bases of most leaves, while the remaining parts of the leaves and leaflets appear green and healthy.
3	Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets.
4	Severe mosaic distortion of two thirds of most leaves and general reduction of leaf size and stunting of shoots.
5	Very severe mosaic symptoms on all leaves, distortion, twisting, misshapen of most leaves accompanied by severe stunting of plants.

10 mins at 4°C. The supernatant was discarded and to the pellet, 500 µl ethanol (70 %) was added. Then tubes were centrifuged at 14,000 rpm for 10 mins at 4°C. Supernatant was discarded and air dried the pellet in 37°C. The pellet was then dissolved in 50 µl nuclease free water or TE buffer (Appendix I) and stored at -20°C.

3.3 ANALYSIS OF DNA

3.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to know the quality of DNA extracted by CTAB method. A 0.8 % agarose gel was prepared using 1X TAE buffer (Appendix II) and ethidium bromide (EtBr) was added to it. About 2 µl of DNA sample was mixed with the gel loading dye (Appendix II) and loaded into the wells. The gel was run at 70V for about 45 mins. Then the gel was visualised under UV light and image was saved using Azure gel documentation system. The quantity of DNA was measured using UV spectrophotometer (Denovix DS 11+ spectrophotometer).

3.4 DETECTION OF VIRUSES USING POLYMERASE CHAIN REACTION

In order to identify the type of viruses present in the samples collected, polymerase chain reaction was performed using different primers synthesised from IDT, USA. The primers synthesised were diluted accordingly and used. The details of the primers used are given in Table 3.

Table 3. Details of primers used for multiplex diagnosis of cassava mosaic viruses (ICMV and SLCMV) from collected samples. 43

Virus target	**Primer name**	**Sequence (5'-3')**	**Amplified region**	**Amplicon size**
Multiplex	ICMV_A_F	GCTGATTCTGGCATTTGTAN	Common region & part of AC1	600 bp (SLCMV) 900 bp (ICMV)
	SLCMV_A_F	TGTAATTCTCAAAAGTTACAGTCN		
	I/SLCMV_A_R	ATATGGACCACATCGTGTCN		
Coat Protein gene	CP-F	GGA TCC ATG TCG AAG CGACCA	Coat protein gene	770 bp
	CP-R	AAG CTT TTA ATT GCT GAC CGA		

PCR reaction mix is listed below:

Components	**Volume**
10X buffer for Dynazyme polymerase II (Thermoscientific)	2.5 µl
dNTP (10 mM)	1 µl
ICMV-A-F (10 pmol/ µl)	0.5 µl
SLCMV-A-F (10 pmol/ µl)	0.5 µl
ICMV/SLCMV-A-R (10 pmol/ µl)	0.5 µl
Taq polymerase (0.05 U/µl)	0.3 µl
Water	12.7 µl
Template DNA	2 µl
Total volume	**20 µl**

PCR was carried out in BioRad C1000 Touch Thermocycler (Germany). PCR was set with initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. Final extension was done at 72°C for 5 min. Controls were kept to distinguish the target products from non-target products and primer dimer. The amplified products along with marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1 %). The gel was viewed under gel documentation system.

3.5 WHOLE GENOME AMPLIFICATION USING ROLLING CIRCLE AMPLIFICATION (RCA)

Using rolling circle amplification mechanism (Haible *et al.*, 2006), the whole genome of selected samples were amplified. This method requires only about 10-20 ng of DNA as template. The reaction mixture was composed of 2 µl of exo-resistant random hexamer primers (500µM), 2 µl of Φ*29* DNA polymerase buffer (10X) and 2 µl of dNTPs (10mM). To this mixture, 2 µl of template DNA was added and mixed well. Then it is denatured for 3 min at 94°C and cooled down to room temperature. After cooling, 4 µl of pyrophosphatase (0.1 U/ µl) and 0.7 µl of *Φ29* DNA polymerase (10 U/ µl) were added and incubated for 18-20 hrs at 30°C. Then heat inactivation is done at 65°C for 10 min. The products of RCA were analysed in 0.8 % agarose gel.

3.5.1 Restriction analysis of RCA products

RCA products were subjected to restriction digestion with *Pst*I and *Bam*HI for obtaining 2.7 kb fragments which represents full length genomic DNA-A and DNA-B respectively. Similarly, pUC19 vector was also restricted with *Pst*I and *Bam*HI separately for ligating the restricted RCA products.

The components of the restriction mixture were optimized as listed below:

Components	Volume
Cutsmart buffer (10X)	2.0 µl
*Pst*I/*Bam*HI (10 U/µl)	1.0 µl
Water	12.0 µl
Template DNA / pUC19 vector DNA	5.0 µl
Total volume	**20 µl**

The digestion process was carried out at 37°C for 1 hr followed by heat inactivation at 65°C for 10 min. The restricted products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1 %). The gel was viewed under gel documentation system.

3.6 CLONING AND TRANSFORMATION

3.6.1 Preparation of competent cells

A loop full of Escherichia coli strain DH5α culture was streaked onto LB agar (Appendix III) plate and incubated for 16-20 hrs at 37°C. From this, a single colony was inoculated into 3 ml LB broth (Appendix IV) and incubated overnight at 37°C with shaking at 180 rpm. About 250 µl of overnight culture was inoculated on 25 ml fresh LB and incubated for 2-4 hrs at 37°C till the O.D reaches 0.5 to 0.6. Then cells were aseptically transferred to centrifuge tube and chilled on ice for 10 mins. The tubes were centrifuged at 4000 rpm for 10 mins at 4°C. Pellet was resuspended into a 25 ml sterile, ice cold 100 mM $CaCl_2$ containing 15 % v/v glycerol. About 200 µl aliquots were made in $CaCl_2$ centrifuge tubes and immediately frozen and stored at -80°C.

3.6.2 Ligation

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The ligation mix for the transformation was prepared as given below: -

Components	**Volume**
Vector pUC19 (restricted with *Pst*I/ *Bam*HI)	2.0 µl
10X ligation buffer	1.0 µl
RCA product (restricted with *Pst*I/ *Bam*HI)	2.0 µl
T4 DNA ligase (10 U/µl)	1.0 µl
Water	4.0 µl
Total volume	**10.0 µl**

The ligation mix was mixed well and incubated overnight at 16°C.

3.6.3 Transformation

The ligation mix was added to tubes containing a volume of about 200 µl competent cells and incubated in ice for 30 mins. Then the mixture was given heat shock at 42° C for 90 secs. Immediately, kept the tubes in ice for 10 mins. Aliquoted 1.8 ml of fresh LB into the tubes having the competent cells ligation mixture incubated at 37°C for 1 hr with shaking at 220 rpm. Finally, the cell suspensions were spread on LA ampicillin /X gal/ IPTG agar plates (AppendixV). The plates were incubated overnight at 37°C and observed for the growth of colonies.

3.6.4 Analysis of recombinant colonies

3.6.4.1 Plasmid DNA isolation from transformed white colonies

White colonies obtained were selected for plasmid DNA isolation and insert was confirmed through restriction and sequencing.

Plasmid isolation was done using the manual method formulated as follows.

The white colonies were inoculated onto 2 ml LB broth containing appropriate antibiotic [(Ampicillin (100 µg/ml)] and incubated overnight with shaking of 180 rpm at 37°C. The grown cultures were then pelletised by centrifuging the tubes at 12,000 rpm for 5 mins. The supernatant was discarded, pellet was resuspended in 200 µl Buffer P1 (Appendix VI) and vortexed. To this, 10 µl activated RNase (10mg/ml) was added and kept at 37°C for 5 mins. About 200 µl of Buffer P2 (Appendix VI) was added to the tubes, inverted and kept in ice for 5 mins. Then 200 µl of Buffer P3 (Appendix VI) was added to the tubes, inverted and kept in ice for 10 mins. The tubes were centrifuged at 12,000 rpm for 10 mins. Supernatant was discarded and to the pellet 500 µl of 70 % ethanol was added. The tubes were centrifuged at 12,000 rpm for 5 mins. The pellet was air – dried and resuspended in about 20 µl of nuclease free water, stored at -20° C.

3.6.4.2 Confirmation of recombinant clones using restriction analysis

Restriction digestion using particular enzymes (*Pst*I and *Bam*HI) was performed for the confirmation of insert in the white colonies.

The components of the restriction mixture were optimized as given below:

Components	**Volume**
Cutsmart buffer (10X)	2.0 µl
*Pst*I/ *Bam*HI (10 U/µl)	1.0 µl
Water	15 µl
Plasmid DNA	2.0 µl
Total volume	**20 µl**

Restriction digestion was carried out at 37°C for 1 hr followed by incubation at 65°C for 10 min for enzyme inactivation. The restricted products along with

marker (1 kb plus) from 'Thermo Scientific' were separated on 0.8 % agarose gel. The gel was viewed under gel documentation system.

3.7 CHARACTERISATION OF VIRUSES

3.7.1 DNA sequencing

Plasmid DNA of SLCMV DNA-A, DNA-B and ICMV DNA-A clones were sequenced at SciGenome Pvt. Ltd (Cochin).

3.7.2 Sequence analysis

Full genome sequences obtained were analyzed and blasted using the basic alignment search tools (BLASTn) in the NCBI website. The sequences having more than 90 % identity were selected from NCBI website.

3.8 CONSTRUCTION OF INFECTIOUS CLONES

3.8.1 SLCMV DNA A

3.8.1.1 Cloning of a 1.7 kb bitmer of DNA A from pSLCMV into the binary vector pPZP201

To obtain a 1.7 kb bitmer of DNA A, pSLCMV was double digested with *PstI/Hin*dIII. Digestion with *PstI/Hin*dIII released three fragments of 1.7 kb, 1 kb and 2.7 kb. The 1.7 kb fragment was subcloned into the corresponding sites of the binary vector pPZP201. Screening was performed by blue/white selection.

3.8.1.2 Partial dimer construction

To construct one-and-a-bitmer (partial dimer) of DNA A, pSLCMV was digested with *Pst*I. Then the 2.7 kb fragment obtained were ligated with the 1.7 kb bitmer of DNA A. Blue white screening was done and the white colonies were screened independently on two antibiotics, Ampicillin and Spectinomycin.

3.8.2 SLCMV DNA B

3.8.2.1 Cloning of a 2.3 kb bitmer of DNA B from pSLCMV into the binary vector pPZP201

To obtain a 2.3 kb bitmer of DNA B, pSLCMV was double digested with *Bam*HI/*Kpn*I. Digestion with *Bam*HI/*Kpn*I released three fragments of 2.3 kb, 0.4 kb and 2.7 kb. The 2.3 kb fragment was subcloned into the corresponding sites of the binary vector pPZP201. Screening was performed by blue/white selection.

3.8.2.2 Partial dimer construction

To construct one-and-a-bitmer (partial dimer) of DNA B, pSLCMV was digested with *Bam*HI. Then the 2.7 kb fragment obtained were ligated with the 2.3 kb bitmer of DNA B. Blue white screening was done and the white colonies were screened independently on two antibiotics, Ampicillin and Spectinomycin.

3.9 MOBILISATION OF PARTIAL DIMERS TO *Agrobacterium tumefaciens* (Ach5)

3.9.1 Day 1

Agrobacterium strain, *Ach5* was streaked and incubated at 28°C for 48 hrs to get single colonies on YEB medium (Appendix VII) agar plate.

3.9.2 Day 2

E. coli harbouring pRK2013 was streaked to get single colonies on LB agar plate with 50 µg/ ml of kanamycin. Then, *E. coli* containing the plasmid i.e donor strain was streaked for single colonies on LB agar medium with suitable antibiotic and incubated overnight at 37°C.

3.9.3 Day 3

A plate containing plain YEB agar was prepared. Single colony each from pRK 2013, donor strain and agrobacterium were patched separately on YEB plate very close to each other. Those 3 bacterial strains were mixed well using a sterile loop. The plate was left at 30°C for 12-18 hrs.

3.9.4 Day 4

6 culture tubes each containing 0.9 ml of 0.9 % NaCl were autoclaved and kept ready. Then 6 plates containing AB minimal medium (Appendix VII) with relevant antibiotic i.e of donor as well as recipient were poured and kept ready. After mating, the bacteria on the YEB plate were scrapped and suspended in 0.9 ml of 0.9 % NaCl. A serial dilution was performed by transferring 0.1 ml of the bacterial suspension into 0.9 ml NaCl (10^{-1} dilution). Likewise, 5 dilutions (up to 10^{-6}) were made. About 100 µl of each dilution was added to an AB medium plate with appropriate antibiotic and spread uniformly using a glass rod. The plates were incubated at 30°C for 3-5 days.

3.9.5 Day 5

At one or three dilutions, single colonies appeared on AB plate. These colonies were *Agrobacterium tumefaciens* into which the donor plasmid had been transferred.

3.9.9 Day 6

About 6 to 8 colonies of *Agrobacterium tumefaciens* were again streaked to single colonies on AB minimal medium containing suitable antibiotic. Single colonies were again seen after 4 days. Then these colonies were patched and maintained as a master plate.

3.10 EXTRACTION OF TOTAL DNA FROM *Agrobacterium tumefaciens*

Total DNA was isolated from *A. tumefaciens* cells as described by Chen and Kuo (1993).

Culture containing cells were pelletised by centrifugation at 12,000 rpm for 5 mins. The tubes were vortexed and resuspended in 200 µl lysis buffer (Appendix VIII) by gentle tapping. About 66 µl of 5M NaCl was added to each tube, mixed well by inversion and kept at -20°C for 10 mins. The tubes were centrifuged at 12,000 rpm for 10 mins. The supernatant was transferred and equal volume of chloroform was added. The tubes were mixed well,

centrifuged at 12,000 rpm for 5 mins and upper phase taken in another tube. The DNA was precipitated with 2.5 volume of 95 % ethanol and left at -20° C for overnight incubation. Centrifuged at 12,000 rpm for 10 mins and to the pellet, 500 µl of 70 % ethanol was added and centrifuged at 12,000 rpm for 5 mins. Pellet was air dried and dissolved in 50 µl of nuclease free water.

3.11 CONFIRMATION OF THE TRANSCONJUGANTS

3.11.1 Polymerase chain reaction (PCR)

PCR was done with CP and MP primers specific to DNA A and DNA B components.

3.11.2 Southern blotting

3.11.2.1 DIG DNA labelling and detection

DNA was isolated from *Agrobacterium* transformants of DNA A and DNA B and recipient strain. Partial dimers digested and linearized with *SalI* were used as a probe to hybridize the respective expected fragment of transformants. *Agrobacterium* total DNA (1.0 µg) was digested with appropriate restriction endonuclease and was electrophoresed in a 0.8 % agarose gel in 1X TBE buffer. The gel was soaked in 250 ml denaturation solution (Appendix IX) in a glass tray and gently shook for 60 mins on a rocker platform. It was washed three times with sterile double distilled water and soaked in 250 ml neutralization solution (Appendix IX) for 45 mins on a rocker platform. The gel was subjected to capillary blotting in saline sodium citrate buffer (20X SSC) (Appendix IX) (as described by Sambrook and Russell, 2001) for the transfer of DNA to the nylon membrane. The transfer was kept overnight. The membrane was rinsed with 2X SSC for 20 sec. The DNA was fixed to nylon membrane by UV-cross linking and baking at 80°C for 30 mins under vacuum.

3.11.2.2 DIG labelling

The probe DNA was prepared, denatured by heating for 5 mins in a boiling waterbath and kept on ice. To the tube containing probe DNA, 2 µl each of

hexanucleotide mix and dNTP mix, 1 µl of Klenow enzyme were added and kept for incubation at 37°C for 15 hrs. Then 2 µl of 0.2 M EDTA (pH 8.0) was added to the tube. After that 2.5 µl of 4 M LiCl and 75 µl of 100 % ethanol were added to the tube and kept overnight at -20° C for precipitating the DNA. On the next day, tubes were centrifuged at 12,000 rm for 10 mins at 4°C. To the pellet, 50 µl of 70 % ethanol was added and centrifuged at 12,000 rpm for 5 mins. The pellet was air dried and dissolved in 50 µl nuclease free water.

3.11.2.3 Hybridisation

The UV cross linked blot was placed inside hybridization bottle using Fischer forceps and pre-warmed (65^0 C) pre-hybridization solution (Appendix IX) was added. Pre-hybridization was carried out for 30 min at 65^0 C in the hybridization incubator (GoldSIM, India). The probe was denatured by boiling for 5 mins and cooled rapidly on ice. Pre-hybridization solution was decanted, replaced with fresh solution and denatured probe was added. Hybridization was performed overnight at 65^0 C.

3.11.2.4 Post-hybridisation washes

The temperature was kept at 65^0 C and salt concentration was decreased in subsequent washes. Hybridization solution was removed and blot was washed successively with 2X SSC / 0.1% SDS, 0.5X SSC / 0.1 % SDS and 0.1X SSC / 0.1 % SDS. Each wash was done at 65^0 C for 30 min in the hybridization incubator.

The membrane was washed for 5 mins in 20 ml wash buffer (Appendix IX). Then it was incubated for 1 hr in 80 ml of 1X blocking solution (Appendix IX). The anti-DIG AP conjugate was diluted in 1:10000 ratio in blocking solution. Membrane was incubated for 30 mins in 20 ml antibody solution and washed two times with 80 ml washing buffer for 15 mins. The membrane was then equilibrated in 40 ml of detection buffer (Appendix IX). The substrate, CSPD was diluted in 1 :100 ratio. Membrane was exposed to about 2 ml of

substrate in a polythene bag for 5 mins under dark conditions. The substrate solution was spread by rolling a test tube over the membrane.

Using Whatman No.1 filter paper, the membrane was dried. The blot was exposed to X-ray film (Konica type AX) in an exposure cassette with an intensifying screen and kept in -70^0 C for 30 mins. The exposed X-ray film was brought to room temperature and developed using the developer and fixer in dark room (KONICAFIX, NIPPON photographics India Ltd., India).

3.12 AGROINOCULATION

Agroinoculation was performed according to Mahajan *et al.* 2011

3.12.1 Raising healthy *Nicotiana benthamiana* plants

The seeds were collected from healthy *N. benthamiana* plants and sowed in fertile soil. After about 2 weeks, two to three leaves staged seedlings were planted in separate pots and kept in plant growth chamber. These plantlets were used for the agroinoculation studies.

3.12.2 Agroinoculation solution preparation

3.12.2.1 Day 1: Preculture

Single colony of *Agrobacterium* strains with infectious clones (SLCMV DNA A and SLCMV DNA B) were inoculated into 3 ml medium and incubated overnight at 28°C.

Components	**Volume**
AB Minimal Medium	3ml
Streptomycin	300 mg/l
Spectinomycin	100 mg/l

3.12.2.2 Day 2: Main culture

2 µl of the overnight cultures used to inoculate 100 ml medium and incubate at 28°C for overnight.

Components	**Volume**
AB Minimal Medium	100 ml
Streptomycin	300 mg/l
Spectinomycin	100 mg/l

3.12.2.3 Day 3:

When the OD reached A_{600}=1, the cells were harvested by centrifugation at 3000 rpm for 10 min at 25°C and the pellet was re-suspended in equal volume of AB minimal medium with 100 µM acetosyringone.

About 10 µl of this culture was injected into the stem of the plant with a G30 syringe needle. The plants were kept in a growth chamber maintained in controlled conditions (16 hrs light/ 8 hrs dark cycles at 28^0C).

After 10 days, symptoms were duly noted down.

RESULTS

4. RESULTS

The results of the study entitled "Development of infectious clones of cassava mosaic virus and their validation" conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2017-2018 are presented in this chapter.

4.1 COLLECTION OF INFECTED LEAF SAMPLES

Cassava mosaic virus infected leaf samples were collected from the fields in ICAR-CTCRI, Thiruvananthapuram. The leaf samples were classified according to the symptom scale of Hahn *et al.* (1980). Out of the 39 samples randomly collected, 8 samples shown scale 1, 5 shown scale 2, 13 shown scale 3, 9 shown scale 4 and 4 samples shown scale 5 of very high mosaic symptoms having distorted leaves with stunting of plants. Representative samples are shown in Fig. 7.

4.2 PLANT DNA ISOLATION

Plant DNA was isolated from the cassava leaf samples by CTAB method. The quality and quantity were analysed using agarose gel electrophoresis and spectrophotometer readings respectively. The gel image showing good quality DNA obtained as bright bands are shown in Fig. 8.

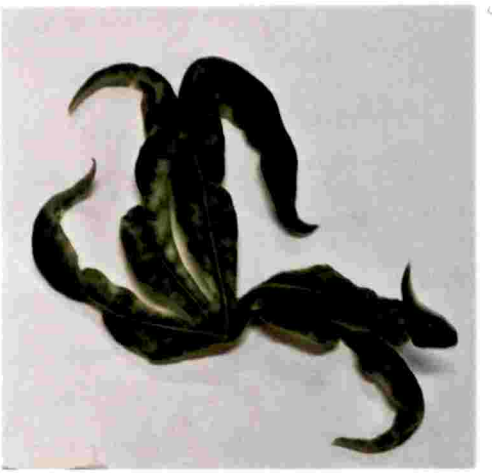
4.3 MOLECULAR DETECTION OF VIRUSES USING POLYMERASE CHAIN REACTION

4.3.1 Multiplex PCR for detection and differentiation of ICMV and SLCMV

Multiplex PCR was performed for the detection and differentiation of ICMV and SLCMV in the infected samples.

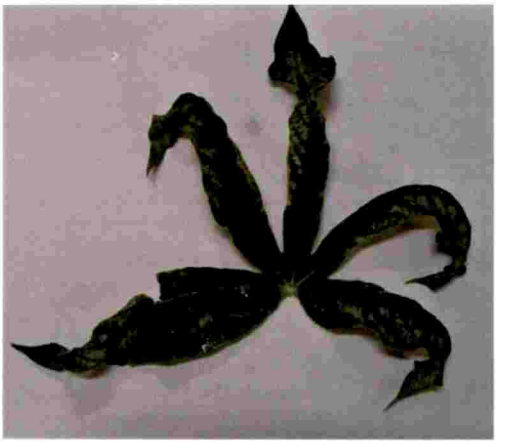
Of the 39 samples collected, 3 samples had only ICMV infection, 25 samples had only SLCMV infection and 11 samples which did not show symptoms had neither ICMV nor SLCMV infection (Table. 4). The gel images showing multiplex PCR results of the samples collected during the survey were shown in Fig. 9.

Variety name: Deevan
Symptom scale: 5



Variety name: H226
Symptom scale: 3

Variety name: Sree Swarna
Symptom scale: 4



Variety name: Sree Vijaya
Symptom scale: 2

Variety name: Sree Jaya
Symptom scale: 2

Variety name: Sree Athulya
Symptom scale: 3

Fig. 7 Representative samples showing different grades of cassava mosaic disease symptoms

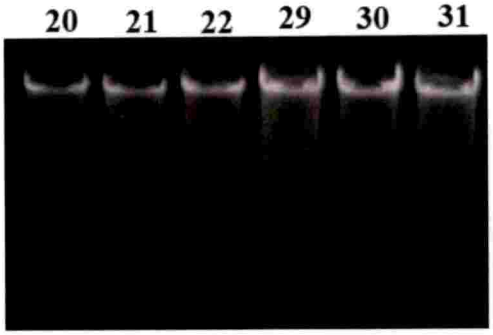


Fig. 8 Good quality DNA isolated from cassava mosaic infected leaf samples. 20- Deevan, 21- Sree Jaya, 22- Vellayani Hraswa, 29- H165, 30- Sree Apoorva, 31- Sree Vijaya.

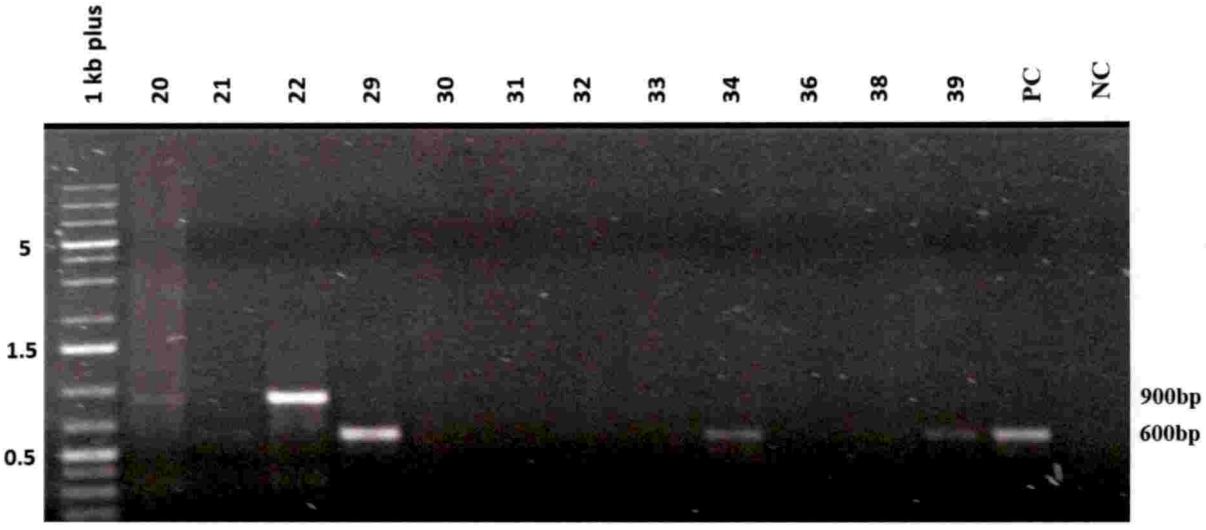


Fig. 9 Gel image showing PCR amplified products from CMD samples collected using multiplex primers. 20- Deevan, 21- Sree Jaya, 22- Vellayani Hraswa, 29- H165, 30- Sree Apoorva, 31- Sree Vijaya, 32- Andhra sample1, 33- Andhra sample2, 34- H226, 36- Unknown, 38- Sree Athulya, 39- Unknown

Table 4. PCR results of collected samples

Sample no.	PCR results		Sample no.	PCR results	
	ICMV	SLCMV		ICMV	SLCMV
1	-	-	21	-	+
2	-	+	22	+	-
3	-	+	23	-	+
4	-	-	24	-	+
5	-	+	25	-	-
6	-	+	26	-	+
7	-	+	27	-	+
8	-	+	28	+	-
9	-	-	29	-	+
10	-	+	30	-	-
11	-	+	31	-	-
12	-	+	32	-	-
13	-	+	33	-	-
14	-	+	34	-	+
15	-	+	35	-	-
16	-	+	36	-	-
17	-	+	37	-	-
18	-	+	38	-	+
19	-	+	39	-	+
20	+	-			

4.4 WHOLE GENOME AMPLIFICATION OF SLCMV/ICMV

The whole genome amplification of SLCMV and ICMV from sample no. 27, 28 respectively, was done using rolling circle amplification (RCA) technique. The RCA products were obtained as a shear of concatemers when run on 0.8% agarose gel. The gel image of RCA products is shown in the Fig. 10.

4.5 RESTRICTION ANALYSIS OF RCA PRODUCTS

While analysing DNA-A and DNA-B genomes of the available ICMV and SLCMV isolates from NCBI, it was found that *Pst*I restriction site is present in DNA-A, but absent in DNA-B. Similarly, *Bam*HI restriction site is present in DNA-B, but absent in DNA-A. So, these two enzymes were used separately to digest RCA amplified products for generating whole genome fragments (2.7 kb) of DNA-A and DNA-B (Fig. 11 and 12).

4.6 TRANSFORMATION

The pUC19 vector was restricted with *Pst*I and *Bam*HI for ligating them with whole genome fragments of DNA-A and DNA-B respectively. After ligation, transformation process was done. Totally, 16 white colonies were obtained in DNA-A clone of SLCMV, while 13 white colonies were obtained in DNA-B clone of SLCMV. In case of ICMV, 11 white colonies obtained in DNA-A clones and about 4 white colonies were obtained in DNA-B clones.

4.6.1 Confirmation of recombinant clones using restriction analysis

The DNA A of SLCMV clones were restricted using restriction enzymes such as *Pst*I, *Hin*dIII, *Eco*RV, *Sac*I separately. pSLCMV A7 was the clone obtained and its digestion pattern is shown in the Fig. 13. The pSLCMV A7 clone was first sequenced by M13 forward and reverse primer. M13 forward gave the sequence from 1430 to 2644 bp while M13 reverse gave the sequence from 223 to 1441 bp. In order to get the remaining sequences, another primer, KVP667 was used and it gave the sequence from 2281 to 2746 and 1 to 702 bp. Then all the

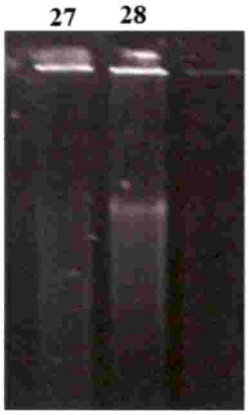


Fig. 10 Gel image showing RCA products of sample no. 27 (SLCMV) and 28 (ICMV).

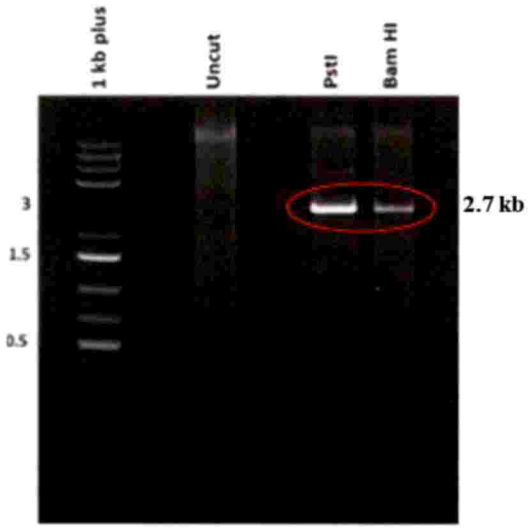


Fig. 11 Gel image for restriction analysis of RCA amplified products of SLCMV (A & B).

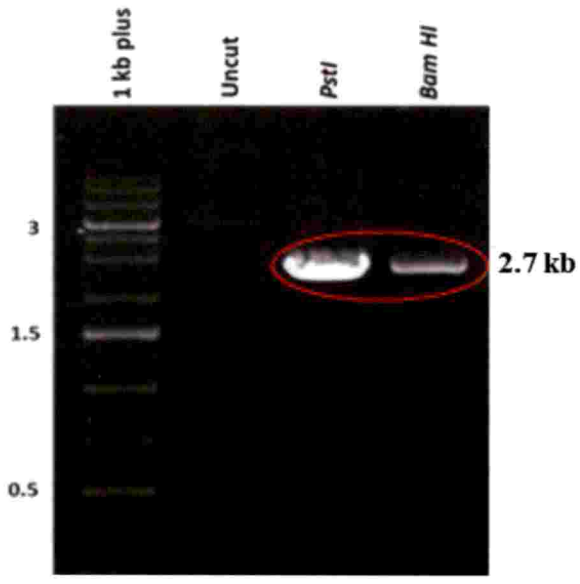
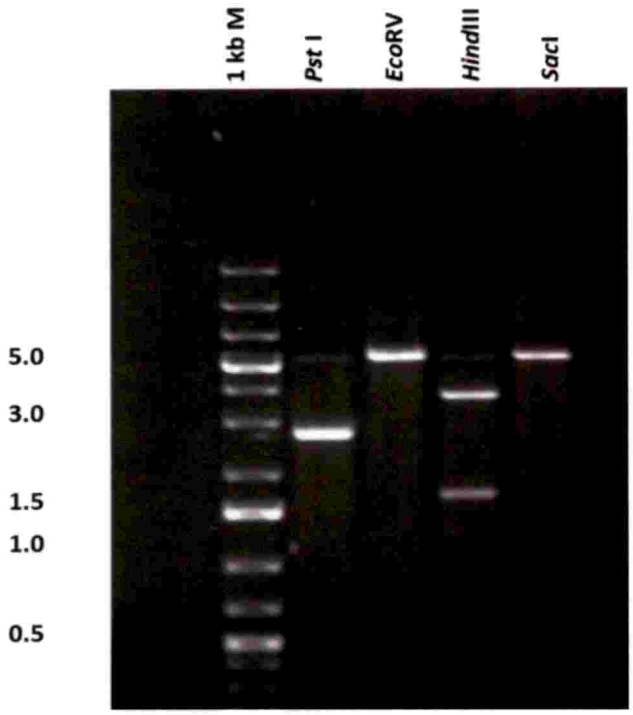
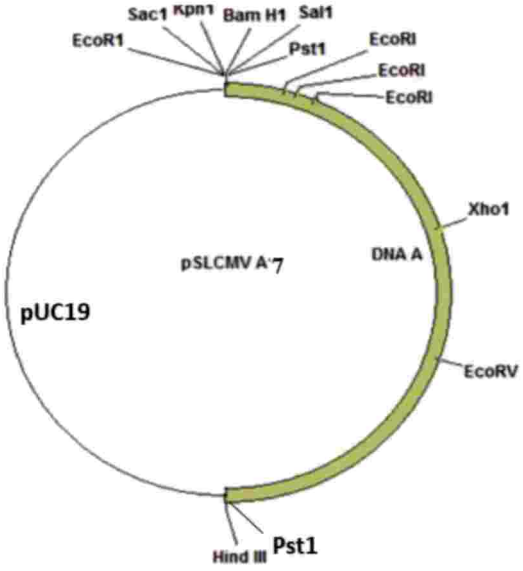


Fig. 12 Gel image for the restriction analysis of RCA amplified products of ICMV (A & B).



(a)



(b)

Enzymes	Sizes (kb) DNA A
Pst I	2.7+2.7
*Hind*III	2.7+1.6
*Eco*RV	5.6
*Sac*I	5.6

(c)

Fig. 13 Construction of pSLCMV A7. (a) Restriction digestion pattern of pSLCMV A7. (b) Restriction map of pSLCMV A7 (c) The sizes of restriction fragments of pSLCMV A7

sequences were aligned manually and 2746 bp sequence of pSLCMV A7 was obtained (Fig. 14).

Similarly, DNA A of ICMV clones were restricted using restriction enzymes such as *Pst*I, *Eco*RV. DNA B of ICMV clones were restricted using restriction enzymes such as *Eco*RI and *Hin*dIII and could not get the proper pattern, attempted further and unable to get the proper clone. pICMV A5 and A6 were the two clones obtained and their digestion pattern is shown in the Fig. 16. The pICMV A5 clone was first sequenced by M13 forward and reverse primer. M13 forward gave the sequence from 226 to 1441 bp while M13 reverse gave the sequence from 1436 to 2528 bp. In order to get the remaining sequences, another primer ICMVA2 was designed and used for sequencing. It gave the sequence from 1 to 358 bp and 2485 to 2739 bp. Then all the sequences were aligned manually and 2739 bp sequence of pICMV A5 was obtained (Fig. 17).

The DNA B clones of SLCMV were restricted using restriction enzymes such as *Bam*HI, *Hin*dIII, *Eco*RV, *Eco*RI, *Kpn*I separately. pSLCMV B2 was the clone obtained and its digestion pattern is shown in the Fig. 21. The pSLCMV B2 clone was first sequenced by M13 forward and reverse primer. M13 forward gave the sequence from 1429 to 2631 bp while M13 reverse gave the sequence from 219 to 1430 bp. In order to get the remaining sequences, another primer SLCMVB2 was designed and used for sequencing. It gave the sequence from 2239 to 2736 bp only. So, another primer KVP580 was used for sequencing and it gave the sequence from 1 to 980 bp. Then all sequences were aligned manually and 2738 bp sequence of pSLCMV B2 was obtained (Fig. 22).

The cloned SLCMV and ICMV isolates were aligned with already established SLCMV and ICMV sequences and identified maximum sequence identity using BLASTn search program (http://www.ncbi.nlm.nih.gov/BLAST) (Fig. 15, 18 and 23). Multiple sequence alignment was performed by using CLUSTALW program (Thompson *et al.*, 1994). For ORF identification, NCBI ORF finder was used. Phylogenetic trees were constructed by the neighbour joining (Saitou and Nei 1987) and bootstrap (Felsenstein 1985) options of

ACCGGATGGCCGCGCCGCCCGCTTTGTGGTGGACCCCCCCCCCACGTGGAGATGTCCCCCAT
TCAGAACGCTCCCTCAAAGCCTGTATAGTTGTGGTCCCTCTTTAAGTACTTGCTCAGCAAGT
TGTAATCTGCACAATGTGGGACCCTTTGGTAAACGAGTTCCCGGATTCAGTTCACGGTTTCC
GGTGTATGCTTGCCGTGAAATATCTTCAGTTAGTTGAAGGTACTTATTCCCCCGATACACTC
GGTTACGATTTAATCAGAGATTTGATCTCTGTCATCAGGGCCAAAAATTATGTCGAAGCGAC
CAGCAGATATCATCATTTCAACTCCCGCCTCGAAGGTTCGTCGCCGTCTGAACTTCGACAGC
CCATACAGCAGTCGTGCTGCTGTCCCCACTGTCCGCGTCACAAAAAGACAAGCCTGGACAAA
CAGGCCCATGAATCGGAAGCCCAGGTGGTACAGGATGTTCAAAAGCCCAGATGTTCCTAGGG
GATGTGAAGGCCCATGTAAGGTTCAATCGTTTGAGTCCAGACACGATGTGGTCCATATAGGT
AAGGTCATGTGCATCTCTGATGTCACTCGTGGAGTTGGGCTTACTCATCGCGTGGGTAAGAG
GTTTTGCGTTAAGTCCGTTTATATCCTGGGTAAGATATGGATGGATGAAAATATTAAGACCA
AGAATCATACGAATAGTGTGATGTTCTTCCTTGTAAGGGATCGTAGGCCTGTTGATAAGCCC
CAGGATTTTGGTGAAGTGTTTAATATGTTCGATAATGAACCTAGTACAGCTACGGTGAAGAA
CATGCATCGTGATCGTTATCAAGTCCTCAGGAAGTGGAGTGCCACTGTCACTGGTGGTCAGT
ATGCGAGCAAGGAACAGGCTTTAGTTAGGCGTTTTTTTAGAGTTAATAATTATGTTGTGTAT
AACCAGCAAGAGGCTGGCAAGTATGAAAATCATACCGAGAATGCATTGATGCTGTACATGGC
GTGTACTCATGCCTCTAACCCTGTATACGCTACGCTGAAGATTAGAATCTACTTCTACGATT
CGGTCAGCAATTAATAAACATTAAATTTTATTAAATTAGACTGCTCAATATTGTCAGTCCCA
GCGATTACATTATATAATACATGTTCTACTGCGTTTACAACCGTATTAATACATATAATCCC
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GCTGTAAGGTCGTCCAGACCTTGAAATCCATCCAGCATTGATGTAGTCCCAACGCTTTCCTC
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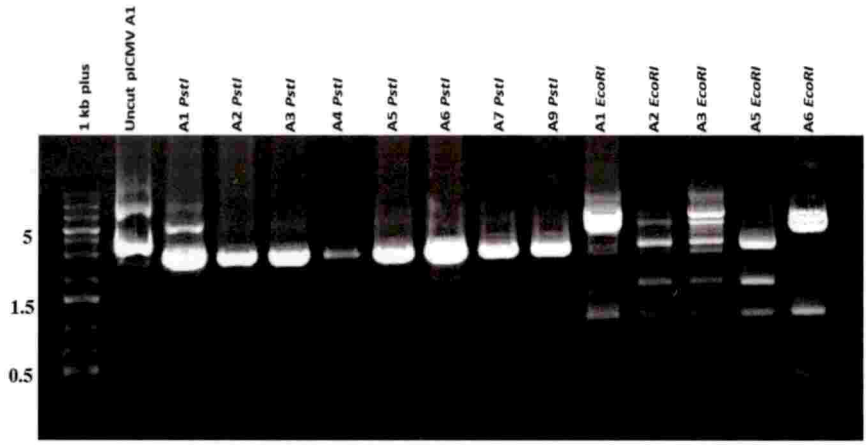
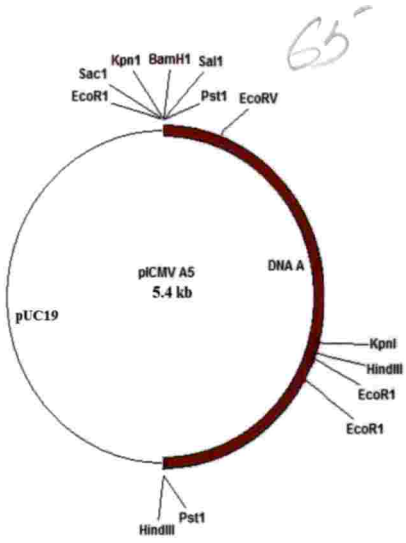
GTCGTGCTGGATGATCTTGAAATAGAGGGGATTTGGAACCTCCCAGATATATACGCCATTCA
TCGCCTGAGCTGCAGTGATGAGTTCCCCTGTGCGTGAATCCATGGTTGTGGCAGTTGATGTG
CACGTAGTATGAGCAGCCGCAATTGAGGTCTACTCTCCGTCGCCGAATGGCCTTACGCTTAG
CTGCCCTGTGTTGGACCTTGATTGGAACCTGAGTAGAGCGGCTCGCTGAGGGAGATGAAGGT
CGCATTCTTCAGAGCCCAAGCCTTCAATGCGCTATTCTTCGCCTCGTCAAGGAATTCTTTAT
AGCTGGAATTGGGCCCAGGATTGCAGAGGAAGATAGTGGGAATTCCCCCTTTAATTTGAACT
GGCTTCCCGTACTTGGTGTTTGACTGCCAGTCTCTTTGGGCCCCCATGAATTCCTTAAAGTG
CTTTAGATAGTGCGGATCTACGTCATCAATTACGTTGTACCATGCATCATTGCTGTAAACCC
TAGGACTCAAGTCCAGATGTCCACACAGATAATTGTGTGGACCTAATGACCTAGCCCACATT
GTTTTGCCTGTACGACTATCGCCCTCGATGACTATACTATTAGGTCTCAAAGGCCTCGCAGA
GGCACCCATGACGTTCTCTGACACCCACTCCTCAAGTTCATCTGGAACTTGGTCAAATGAAG
AGGCTGAGAAGGGAGACACATATACCTCGGGAGGAGGTGTAAAAATCCTATCTAAATTAGCA
TTTAGATTATGAAATTGTAAAACATAATCCTTGGGTGCTAACTCCTTAATGACTCTAAGAGC
CTCTGGCTTACTACCTGTGTTAAGTGCTGCGGCGTAAGCGTCGTTTGCTGTCTGCTGTCCCC
CTCTTGCAGATCTTCCATCGATCTGAAACTCACCCCAGTCGATGGTGTCTCCATCCTTGTCG
ATGTAGGACTTGACGTCTGAGCTGGACTTAGCGCTCTGTATGTTGGGGTGGAAACTGGTGCT
ACTGCTTGGGTGTACACAATCGAATTGCCGATTGTTTGTGATTGTGAGCTTCCCTTCGAACT
GAAGCAGAGCATGGAGGTGAGGTTCCCCATTCTGATGGAGTTCTCTGCAAATTTTAATAAAT
TTGATGTTTGTCGGAAGATTCAAGCTTCGGAAGAACTCGAGTAAGTGTTCTTTGGTGAGAGA
ACACTTAGGGTATGTGAGGAATATATTTTTGGACTGAATTCTGAATCGTGGGTTTCTCATCT
TGGACTCGGTCAATTGGAGACACCCCTGAGCATGTCTCTAGTGAATTGGAGACAATATATAT
GTGTCTCCAAATGGCATTCTTGTAATTCTCAAAAGTTTCAAATCCGGATTTGAAATCCAAAA
GCGGCCATCCGTATAATATT

Fig. 14 The virion-sense strand nucleotide sequence pSLCMV A7 (2746bp)



(a)



(b)

Enzymes	Sizes (kb) DNA A
*Pst*I	2.7+2.7
EcoRI	3+1.6+1 or 0.2+0.1+0.9+3.3

(c)

Fig. 16 Construction of pICMV A5. (a) Restriction digestion analysis of clones A1 to A7 with *Pst*I and *Eco*RI. (b) The restriction map of pICMV A5. (c) The sizes of restriction fragments of pICMV A5 with *Pst*I and *Eco*RI

ACCGGATGGCCGCGCCCCCCCGCTTTGTGGTGGACCCCCCCCCACGTGGAGATGTCCCCCAC
TCAAAACGCTCCCTCAAAGCCTGTATAGTTGTGGTCCCTCTTTAAGTACTTGCTCAGCAAGT
TGTAATCTGCACAATGTGGGACCCTTTGGTAAACGAGTTCCCTGATTCAGTTCACGGTTTCC
GGTGTATGCTTGCCGTGAAATATCTTCAGCTAGTTGAAGGTACTTATTCCCCCGATACACTC
GGTTACGATTTAATCAGAGATTTGATCTCTGTCATCAGGGCCAAAAATTATGTCGAAGCGAC
CAGCAGATATCATCATTTCAACTCCCGCCTCGAAGGTTCGTCGCCGTCTGAACTTCGACAGC
CCATACAGCAGTCGTGCTGCTGTCCCCACTGTCCGCGTCACAAAAAGACAAGCCTGCACAAA
CAGGCCCATGAATCGGAAGCCCAGGTGGTACAGGATGTTCAAAAGCCCAGATGTTCCTAGGG
GATGTGAAGGCCCATGTAAGGTTCAATCGTTTGAGTCCAGACACGATGTGGTCCATATAGGT
AAGGTCATGTGCATCTCTGATGTCACTCGTGCAGTTGGGCTTACTCATCGCGTGGGTAAGAG
GTTTTGCGTTAAGTCCGTTTATATCCTGGGTAAGATATGGATGGATGAAAATATTAAGACCA
AGAATCATACGAATAGTGTGATGTTCTTCCTTGTAAGGGATCGTAGGCCTGTTGACAAGCCC
CAGGATTTTGGTGAAGTGTTTAATATGTTCGATAATGAACCTAGTACAGCTACGGTGAAGAA
CATGCATCGTGATCGTTATCAAGTCCTCAGGAAGTGGAGTGCCACTGTCACTGGTGGTCAGT
ATGCGAGCAAGGAACAGGCTTTAGTTAGGCGTTTTTTTAGAGTTAATAATTATGTTGTGTAT
AACCAGCAAGAGGCTGGCAAGTATGAAAATCATACCGAGAATGCATTGATGCTGTACATGGC
GTGTACTCATGCCTCTAACCCTGTATACGCTACGCTGAAGATTAGAATCTACTTCTACGATT
CGGTCAGCAATTAATAAACATTAAATTTTATTAAATTAGACTGCTCAATACTGTCAGTCCCA
GCGATTACATTATATAATACATGTTCTACTGCGTTTACAACCGTATTAATACATATAATCCC
TAATCTATCGAGGTATTTTAATACTTGGGTCATAAATACCCTCAAGAAACGCCAGGTCTGAG
GCTGTAAGGTCGTCCAGACCTTGAAATCCATCGAGCATTGATGTAGTCCCAACGCTTTCCTC
AGGTTGTGGTTGAAGCGTATCTGGATGGTTATTATGTCCCACGGCCTGTTGAACGGCCGGCT
GTCGTGCTGAATGATCTTGAAATAGAGGGGGTTTGTTACCTCCCAGATAAAGACGCCATTCA
TCGCCTGAGCTGCAGTGATGAGTTCCCCTGTGCGTGAATCCATGGTTGTGGCAGTTAATGTG
GACGTAGTATGAGCACCCGCAGTTGAGATCTACCCTTTTACGCCGGATGGCTTTACGCTTAG
CAGCTCTGTGTTGGACCTTGATTGGTACCTGAGTATAGTGGTCCTTCGAGGGAGATGAAGGT
CGCATTTTTTAGAGCCCAAGCTTTTAATGCGCTATTCTTTTCCTCGTCCAGGAATTCTTTAT
AGCTGGAATTGGGGCCTGGATTGCAGAGGAAGATAGTGGGAATACCTCCTTTAATTTGAACT
GGCTTTCCGTACTTGGTGTTTGATTGCCAGTCCCTCTGGGCCCCCATGAATTCTTTGAAGTG
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TAGGACTTAAATCCAGGTGTCCACATAGGTAATTATGTGGACCCAATGCACGGGACCACATG
GTCTTGCCCGTCCGACTATCGCCTTCGATTACGATTGATTGTGGTCTCAAAGGCCGCGCAGC
GGCACCCATCACGTTTTCGTGGAACCACTCGTCGAGTTCTTCTGGAACTCGGTCAAACGAAG
AGAGAGGGAAAGGGTTCTCATATGGAGGTGGTGGTTTTGTAAAAATCCGATCCAGGTTTGAG
CTGATGTGATGGAAGTCCCTAAGATAATCCCTAGGTGCTAATTCCCTAAGGATCTTAAGAGC
CTCTGATTTACTGCCGCTGTTAAGTGCTGCGGCGTAAGCGTCGTTTGCAGATTGTTGACCCC
CTCTGGCCGATCGTCCATCGATCTGAAATGTTCCCCATTCCAAGGTATCACCGTCCTTGTCG
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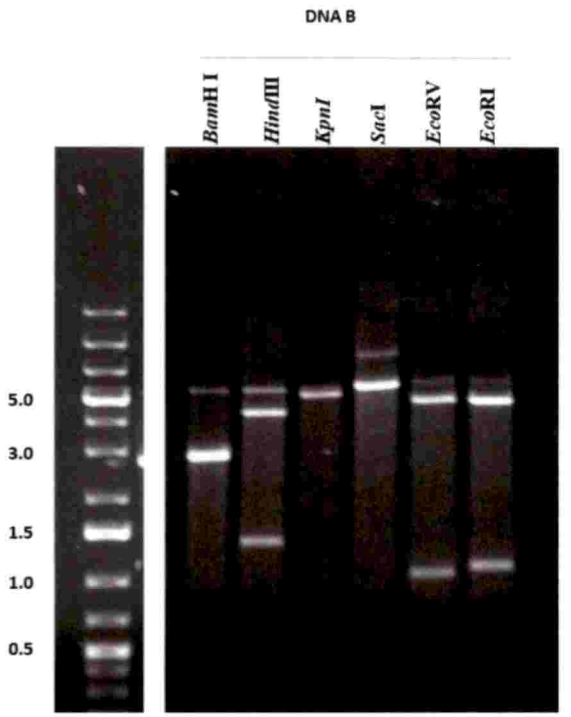
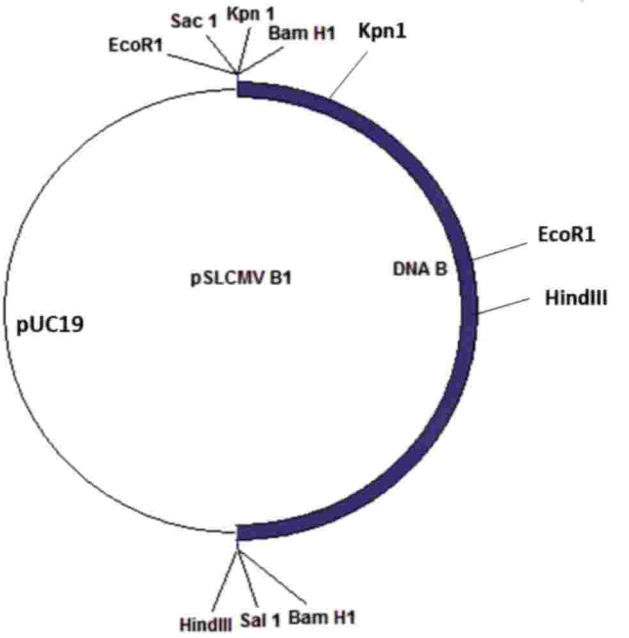
CCTGGTTGGTGATACCAGGTCGAAGAATCGCTGATTCTGGCATTTGTATTTGCCTTCGAACT
GGATGAGCACGTGCAGATGAGGCTCCCTATTCTCATGTAGCTCCCTGCATATTTTGATGAAT
TTAGTGTTTGTAGGTGTTTGAAAGTTCCTAATTTGAGAGAGAGCCTCTTCTTTAGTTAAGGA
GCATCGAGGGTAAGTGAGGAAATAGTTTTTAGCGTTTATTTGAAAGCGCTTAGGTGGTGACA
TTCTCTCTCCTCAATCGGTACTCAGATAGTTTAGCCCCCATATTAGGTACTCAATATATACA
TGAGTACCAAATGGCATAGATGTAAATAATGGGAATATAATTTGAATTCAAAAGCGGCCATC
CGTATAATATT

Fig. 17 The virion-sense strand nucleotide sequence of pICMV A5 (2739bp)



(a)



(b)

Enzymes	Sizes (kb) DNA B
BamHI	2.7+2.7
HindIII	4.2+1.4
EcoRV	4.5+1.1
EcoRI	4.4+1.2
KpnI	5.2+0.4

(c)

Fig. 21 Construction of pSLCMV B2. (a) Restriction digestion pattern of pSLCMV B2. (b) Restriction map of pSLCMV B2 (c) The sizes of restriction fragments of pSLCMV B2

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CCCTATATGTGGATATCTAACCGCATATTGTGTGAATGGATTAAACGTGGCAAGATCATGCCGTT
TATTGGGAACTATATTATTATGCTGTTGTCTATATATATAGGTATGTCGTGTTTCGGAGATCTGC
ACATGGTGGTGGCAGGATGAGAAGAGGTGCCTATACCCCCCGTTCTACTCCATTCCCTCGTGACC
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TGCCTGCCAAAACACGTGCCCTTAGCGATAACAGGGTAGGTGATTATATCAAACTTGTAAATATA
TCATTTACAGGTACAGTGTGTATTAAAAACAGCCAGATGGAATCTGACGGAAGCCCAATGTTGGG
CCTGCATGGGCTGTTTACTTGTGTATTGGTCCCGGATAAGACCCCTCGTATATATTCTGCCACTG
AGCCTTTGATACCTTTCCCACAGTTGTTTGGGTCCATAAACGCGAGCTATGCGGATTTGTCTATA
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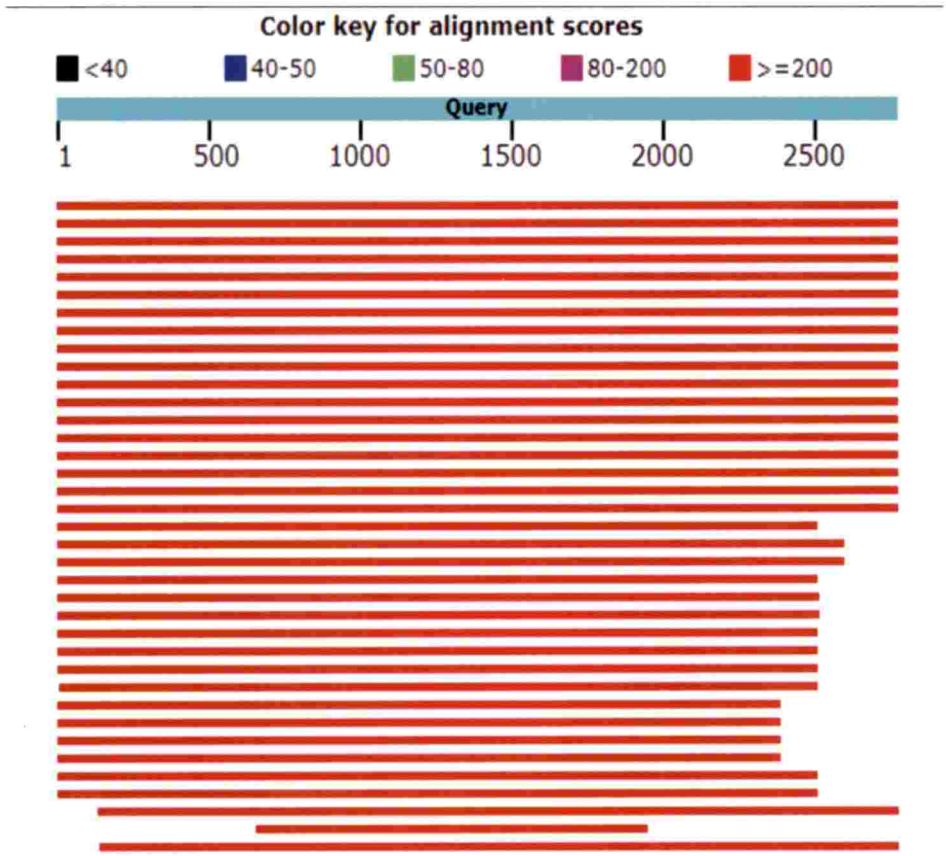
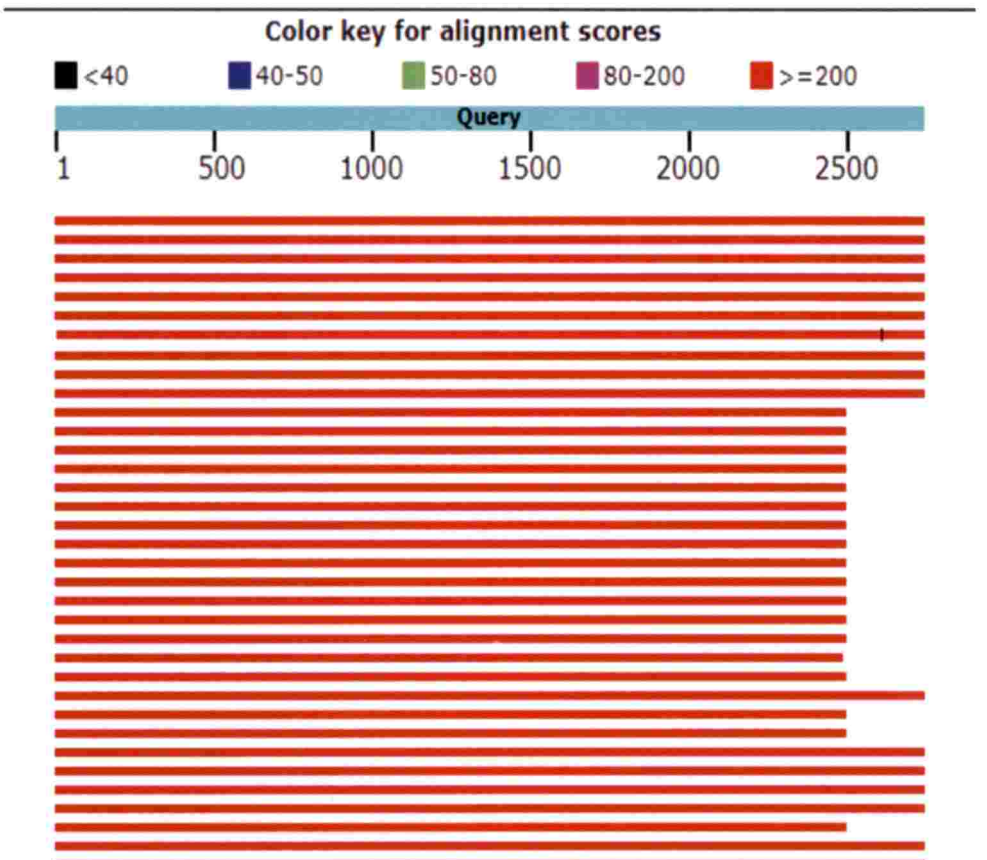
Fig. 22 SEQUENCE OF pSLCMV B2 (1 to 2738)



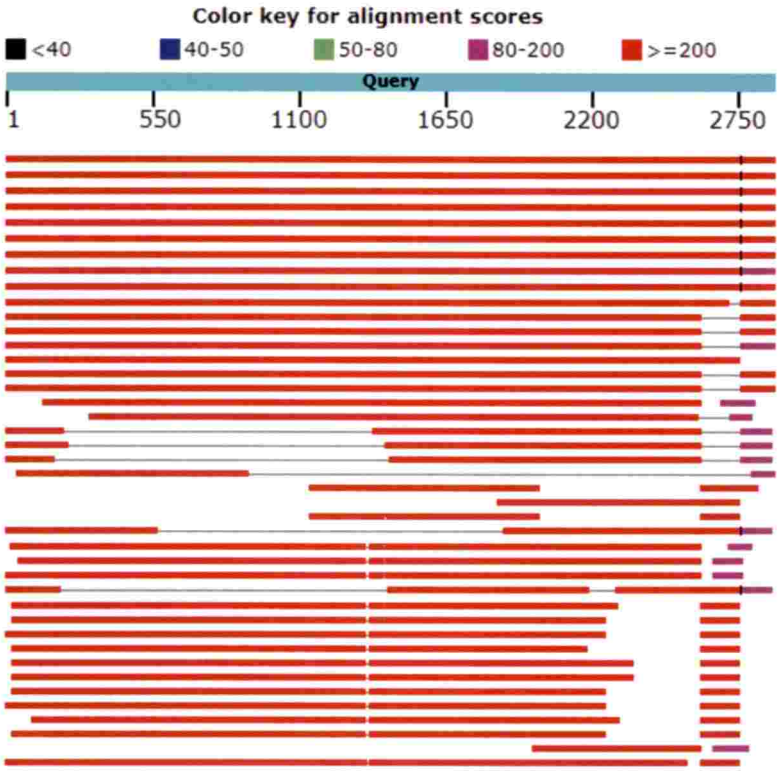
Description	Max score	Total score	Query cover	E value	Ident	Accession
Sri Lankan cassava mosaic virus isolate TVM1 segment DNA-A, complete sequence	4935	4935	100%	0.0	99%	KR611579.1
Sri Lankan cassava mosaic virus isolate TVM3 segment DNA A, complete sequence	4929	4929	100%	0.0	99%	KP455486.1
Sri Lankan cassava mosaic virus isolate SLCMA A segment DNA-A, complete sequence	4902	4902	100%	0.0	99%	KT861468.1
Sri Lankan cassava mosaic virus strain Erode 2011 segment DNA-A, complete sequence	4894	4894	100%	0.0	99%	KU550961.1
Sri Lankan cassava mosaic virus ac1 gene, ac2 gene, ac3 gene, ac4 gene, av1 gene and av2 gene, isolate Kerala 17, complete virus segment	4889	4889	100%	0.0	99%	AJ890225.1
Sri Lankan cassava mosaic virus-India[Attur:2009] segment DNA-A, complete sequence	4885	4885	100%	0.0	99%	KC424490.1
Sri Lankan cassava mosaic virus - [Ker20] segment A, isolate Adivaram	4885	4885	100%	0.0	99%	AJ579307.1
Sri Lankan cassava mosaic virus ac1 gene, ac2 gene, ac3 gene, ac4 gene, av1 gene and av2 gene, isolate Kerala 15, complete virus segment	4883	4883	100%	0.0	99%	AJ890224.1
Sri Lankan cassava mosaic virus genomic DNA, segment A, complete sequence, isolate: VTN6	4879	4879	100%	0.0	99%	LC312131.1
Sri Lankan cassava mosaic virus ac1 gene, ac2 gene, ac3 gene, ac4 gene, av1 gene and av2 gene, isolate Tamil Nadu 7, complete virus segment	4861	4861	100%	0.0	99%	AJ890229.1
Sri Lankan cassava mosaic virus isolate Erode segment DNA-A, complete sequence	4855	4855	100%	0.0	99%	KF898349.1
Sri Lankan cassava mosaic virus segment A, isolate Salem	4830	4830	100%	0.0	98%	AJ607394.1
Sri Lankan cassava mosaic virus ac1 gene, ac2 gene, ac3 gene, ac4 gene, av1 gene and av2 gene, isolate Tamil Nadu 2, complete virus segment	4822	4822	100%	0.0	98%	AJ890227.1
Sri Lankan cassava mosaic virus ac1 gene, ac2 gene, ac3 gene, ac4 gene and av1 gene, isolate Tamil Nadu 6, complete virus segment	4815	4815	100%	0.0	98%	AJ890228.1
Sri Lankan cassava mosaic virus isolate Malappuram segment DNA-A, complete sequence	4723	4723	100%	0.0	98%	KR611577.1
Sri Lankan cassava mosaic virus - India [India:Kerala C4] ac1 gene, ac2 gene, ac3 gene, ac4 gene, av1 gene and av2 gene, isolate:Kerala C4, complete virus segment	4429	4429	100%	0.0	96%	AJ890226.1
Sri Lankan cassava mosaic virus isolate Attur 2 segment DNA A, complete sequence	4137	4137	100%	0.0	94%	KP455484.1
Sri Lankan cassava mosaic virus-[Colombo] DNA-A, complete genome, isolate SLCMV-Col	4023	4023	100%	0.0	93%	AJ314737.1
Indian cassava mosaic virus isolate TVM4 segment DNA-A, complete sequence	3555	3555	90%	0.0	93%	KU308385.1
Indian cassava mosaic virus strain Salem 2011 segment DNA-A, complete sequence	3386	3386	93%	0.0	91%	KU550960.1

Fig. 15 NCBI blast of pSLCMV A7 with already published sequences.



Description	Max score	Total score	Query cover	E value	Ident	Accession
Indian cassava mosaic virus-[Maharashtra] DNA-A, complete genome, isolate ICMV-Mah	4322	4322	100%	0.0	95%	AJ314739.1
Indian cassava mosaic virus isolate TVM4 segment DNA-A, complete sequence	4274	4274	100%	0.0	95%	KU308385.1
Indian cassava mosaic virus isolate Mah-2 segment DNA A, complete sequence	4244	4244	100%	0.0	95%	AY730035.2
Indian cassava mosaic virus-[Ker2] DNA A, isolate Adivaram 2	4120	4120	100%	0.0	94%	AJ575819.1
Indian cassava mosaic virus strain Salem 2011 segment DNA-A, complete sequence	4061	4061	100%	0.0	93%	KU550960.1
Jatropha curcas mosaic virus-[Dha] isolate Dharwad segment DNA-A, complete sequence	3941	3941	100%	0.0	93%	GQ924760.1
Indian cassava mosaic virus encoding AR0 complete CDS	3940	4184	99%	0.0	94%	Z24758.1
Jatropha curcas mosaic virus isolate ICMV-IN SG 12 segment DNA-A, complete sequence	3927	3927	100%	0.0	93%	
Jatropha curcas mosaic virus isolate JC-3 segment DNA-A, complete sequence	3880	3880	100%	0.0	92%	JN692494.1
Jatropha curcas mosaic virus-[Jalgaon] segment DNA-A, complete sequence	3644	3644	100%	0.0	91%	JF496657.1
Sri Lankan cassava mosaic virus isolate TVM1 segment DNA-A, complete sequence	3415	3415	90%	0.0	91%	KR611579.1
Sri Lankan cassava mosaic virus ac1 gene, ac2 gene, ac3 gene, ac4 gene, av1 gene and av2 gene, isolate Kerala 17, complete virus segment	3408	3408	90%	0.0	91%	AJ890225.1
Sri Lankan cassava mosaic virus isolate SLCMA_A segment DNA-A, complete sequence	3398	3398	90%	0.0	91%	KT861468.1
Sri Lankan cassava mosaic virus isolate TVM3 segment DNA A, complete sequence	3398	3398	90%	0.0	91%	KP455486.1
Sri Lankan cassava mosaic virus-India[Attur:2009] segment DNA-A, complete sequence	3397	3397	90%	0.0	91%	KC424490.1
Sri Lankan cassava mosaic virus genomic DNA, segment A, complete sequence, isolate: VTN6	3386	3386	90%	0.0	91%	LC312131.1
Sri Lankan cassava mosaic virus strain Erode:2011 segment DNA-A, complete sequence	3386	3386	90%	0.0	91%	KU550961.1
Sri Lankan cassava mosaic virus - [Ker20] segment A, isolate Adivaram	3386	3386	90%	0.0	91%	AJ579307.1
Sri Lankan cassava mosaic virus ac1 gene, ac2 gene, ac3 gene, ac4 gene, av1 gene and av2 gene, isolate Kerala 15, complete virus segment	3384	3384	90%	0.0	91%	AJ890224.1

Fig. 18 NCBI blast of pICMV A5 with already published sequences of ICMV.



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sri Lankan cassava mosaic virus - [Ker20] segment B, isolate Adivaram	4885	5117	100%	0.0	99%	AJ579308.1
Sri Lankan cassava mosaic virus strain Erode 2011B segment DNA-B, complete sequence	4872	5093	100%	0.0	99%	KU550962.1
Sri Lankan cassava mosaic virus isolate SLCMV_B segment DNA-B, complete sequence	4861	5093	100%	0.0	99%	KT861469.1
Sri Lankan cassava mosaic virus isolate Attur 2 segment DNA B, complete sequence	4861	5082	100%	0.0	99%	KP455485.1
Sri Lankan cassava mosaic virus-[Ker4] DNA B, isolate Kattukuda	4857	5073	100%	0.0	99%	AJ575821.1
Sri Lankan cassava mosaic virus genomic DNA, segment B, complete sequence, isolate: VTN6	4850	5082	100%	0.0	99%	LC312130.1
Sri Lankan cassava mosaic virus isolate TVM3 segment DNA B, complete sequence	4831	5047	100%	0.0	99%	KP455487.1
Sri Lankan cassava mosaic virus isolate Erode segment DNA-B, complete sequence	4800	4986	100%	0.0	98%	KF898350.1
Sri Lankan cassava mosaic virus isolate Malappuram segment DNA-B, complete sequence	4573	4788	100%	0.0	97%	KR611578.1
Sri Lankan cassava mosaic virus isolate TVM1 segment DNA-B, complete sequence	4471	4681	98%	0.0	96%	KR611580.1
Indian cassava mosaic virus-[Ker5] DNA B, isolate Koodathai	4431	4641	95%	0.0	97%	AM158955.1
Indian cassava mosaic virus-[Ker6] BV1 gene for nuclear shuttle protein and BC1 gene for movement protein	4425	4635	95%	0.0	97%	AJ512823.1
Indian cassava mosaic virus-[Maharashtra] DNA-B, complete genome, isolate ICMV-Mah	4405	4594	95%	0.0	97%	AJ314740.1
Sri Lankan cassava mosaic virus-[Colombo] DNA-B, complete genome, isolate SLCMV-Col	4383	4383	95%	0.0	96%	AJ314738.1
Indian cassava mosaic virus-[Ker3] DNA B, isolate Muvattupuzzha	4368	4570	95%	0.0	97%	AJ575820.1
Indian cassava mosaic virus isolate TVM4 segment DNA-B, complete sequence	4340	4544	95%	0.0	97%	KU308386.1
Indian cassava mosaic virus isolate Mah-2 segment DNA B, complete sequence	4133	4133	85%	0.0	97%	AY730036.2
Indian cassava mosaic virus BR1 and BL1 genes, complete CDS's	3639	3762	81%	0.0	96%	Z24759.1
Indian cassava mosaic virus recombinant defective DNA B, clone D1B (ICMV-[Ker3]B)	2061	2566	54%	0.0	97%	AM238428.1
Indian cassava mosaic virus recombinant defective DNA B, clone D2B (ICMV-[Ker3]B)	2006	2503	53%	0.0	97%	AM238429.1
Indian cassava mosaic virus recombinant defective DNA (ICMV-[Ker6]B)	1997	2463	51%	0.0	98%	AM231026.1

Fig. 23 NCBI blast of pSLCMV B2 with already published sequences of SLCMV.

CLUSTALW. The phylogenetic trees were drawn using 'njplot' tree drawing program (Perriere and Gony 1996).

All DNA A components contain six ORFs, two on virion-sense strand and four on complementary-sense strand (Table 5). The two ORFs on virion-sense strand *AV1* and *AV2* coded for coat protein and pre-coat protein respectively. Four ORFs present in the complementary-sense strand, *AC1*, *AC2*, *AC3* and *AC4* coded for replication associated protein, transcriptional activator protein, replication enhancer protein and AC4 protein.

The cloned DNA B components contain two ORFs, *BV1* and *BC1* (Table 7). *BV1* situated in virion-sense strand encoded the nuclear shuttle protein and *BC1* situated in complementary-sense strand encoded the movement protein.

Sequence identity matrix showing the relative nucleotide identity of SLCMVA7 and ICMVA5 isolates were shown in Table. 6. The sequence identity matrix of SLCMVB2 isolate was shown in Table. 8. After phylogenetic analysis it was found that the sequence of pSLCMV A7 showed maximum similarity of 99 % with 'SLCMV-[TVM1]' sequence in NCBI (Fig. 19). While the sequence of pSLCMV B2 showed maximum similarity of 99 % with 'SLCMV-[Ker20]' sequence in NCBI (Fig. 24). The sequence of pICMV A5 showed maximum similarity of 95 % with 'ICMV-[Mah]' sequence in NCBI (Fig. 19).

The intergenic region (IR) of the cloned DNA A and DNA B components possess the conserved nonanucleotide sequence, TAATATTAC, which contains the DNA nicking site for the initiation of viral DNA replication. Two 11-nt putative Rep binding motifs (iterons) AATTGGAGACA were present in both SLCMV DNA A and DNA B components, upstream of the nonanucleotide sequence (Fig. 20 (b), 25). In case of ICMV DNA A, two 8-nt putative Rep binding motifs (iterons) GGTACTCA were present upstream of the nonanucleotide sequence (Fig. 20 (a)).

Table 5. Sequence coordinates of the ORFs encoded by DNA As of isolates cloned in this study

ISOLATES	**LENGTH**	**Coordinates of ORFs**					
		AC1	***AC2***	***AC3***	***AC4***	***AV1***	***AV2***
pSLCMV A7	2746	2602-1547	1617-1210	1469-1065	2448-2146	298-1068	1210-1617
pICMV A5	2740	2605-1547	1617-1183	1469-1065	2454-2146	298-1068	138-494

Table 7. Sequence coordinates of the ORFs encoded by DNA B of isolate cloned in this study 74

ISOLATES	LENGTH	Coordinates of ORFs	
		BV1	***BC1***
pSLCMV B2	2738	342-1124	1993-1133

Table 8. Sequence identity matrix showing the relative nucleotide identity of SLCMV B2 isolate

Sequence	**SLCMV_B2**	**SLCMV-Ker20**	**SLCMV-Erode_2011**	**SLCMV-Ker4**	**SLCMV-TVM3**	**ICMV-Ker5**	**ICMV-Mah**	**SLCMV-Colombo**	**JCMV-Jalgaon**	**ALMDV-AIK1**	**CLCAV-SZ5**
SLCMV_B2	ID	0.99	0.99	0.99	0.99	0.97	0.97	0.96	0.83	0.71	0.68
AJ579308.1		ID	0.99	0.99	0.99	0.95	0.95	0.96	0.82	0.7	0.67
KU550962.1			ID	0.99	0.98	0.95	0.95	0.96	0.82	0.69	0.65
AJ575821.1				ID	0.99	0.96	0.95	0.96	0.82	0.7	0.65
KP455487.1					ID	0.95	0.95	0.96	0.82	0.69	0.65
AM158955.1						ID	0.98	0.95	0.84	0.7	0.66
AJ314740.1							ID	0.94	0.84	0.7	0.65
AJ314738.1								ID	0.81	0.69	0.66
JF496656.1									ID	0.77	0.71
MG969497.1										ID	0.78
KY420150.1											ID

Table 6. Sequence identity matrix showing the relative nucleotide identity of SLCMVA7 and ICMVA5 isolates.

Sequence	SLCMV A7	ICMV A5	ICMV-Mah	ICMV-Mah-2	JCMV-Dha	JCMVICMV-IN_SG_12	ICMV-Ker2	ICMV-Salem	ICMV-TVM4	SLCMV-Erode_2011	SLCMV-Ker15	SLCMV-TVM1	SLCMV-Attur_2009	SLCMV-Salem	SLCMV-Tamil_7	SLCMV-Erode	SLCMV-[Ker20]	SLCMV-TVM3	SLCMV-Malappuram	SLCMV-Col	ChiLCV-58SA	CLC_Khokran	ACMV-NG_LI_06	ACMV-DRC6
SLCMV A7	ID	0.87	0.83	0.83	0.84	0.84	0.85	0.87	0.88	0.99	0.99	0.99	0.99	0.98	0.98	0.98	0.99	0.99	0.98	0.93	0.75	0.74	0.72	0.41
ICMV A5		ID	0.95	0.95	0.93	0.93	0.94	0.93	0.95	0.87	0.87	0.87	0.87	0.86	0.87	0.87	0.87	0.87	0.86	0.82	0.73	0.74	0.69	0.4
AJ314739.1			ID	0.99	0.92	0.92	0.92	0.91	0.91	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.84	0.74	0.74	0.7	0.4
AY730035.2				ID	0.91	0.91	0.91	0.91	0.91	0.83	0.83	0.83	0.83	0.83	0.82	0.83	0.83	0.83	0.82	0.83	0.74	0.74	0.7	0.4
GQ924760.1					ID	0.94	0.93	0.92	0.93	0.84	0.84	0.84	0.84	0.84	0.83	0.84	0.84	0.84	0.83	0.81	0.73	0.74	0.69	0.4
JX518289.1						ID	0.92	0.91	0.91	0.84	0.84	0.84	0.84	0.84	0.83	0.84	0.84	0.84	0.83	0.81	0.74	0.74	0.7	0.4
AJ575819.1							ID	0.96	0.94	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.81	0.72	0.74	0.69	0.4
KU550960.1								ID	0.95	0.87	0.87	0.88	0.87	0.88	0.87	0.87	0.87	0.88	0.86	0.83	0.73	0.74	0.69	0.4
KU308385.1									ID	0.88	0.88	0.88	0.88	0.87	0.88	0.88	0.88	0.88	0.87	0.82	0.73	0.74	0.69	0.39
KU550961.1										ID	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.93	0.74	0.73	0.72	0.41
AJ890224.1											ID	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.93	0.74	0.73	0.72	0.41
KR611579.1												ID	0.99	0.98	0.98	0.98	0.99	0.99	0.97	0.93	0.75	0.74	0.72	0.41
KC424490.1													ID	0.99	0.99	0.99	0.99	0.99	0.98	0.93	0.74	0.73	0.72	0.41
AJ607394.1														ID	0.99	0.99	0.99	0.98	0.97	0.93	0.74	0.73	0.72	0.41
AJ890229.1															ID	0.99	0.99	0.98	0.98	0.93	0.74	0.73	0.72	0.41
KF898349.1																ID	0.99	0.98	0.98	0.93	0.74	0.73	0.72	0.41
AJ579307.1																	ID	0.99	0.98	0.93	0.74	0.73	0.72	0.41
KP455486.1																		ID	0.97	0.93	0.75	0.73	0.73	0.41
KR611577.1																			ID	0.93	0.74	0.73	0.72	0.41
NC_003861.1																				ID	0.75	0.73	0.73	0.41
KT984070.1																					ID	0.78	0.71	0.4
AY456683.1																						ID	0.7	0.39
EU685320.1																							ID	0.43
FN668378.1																								ID

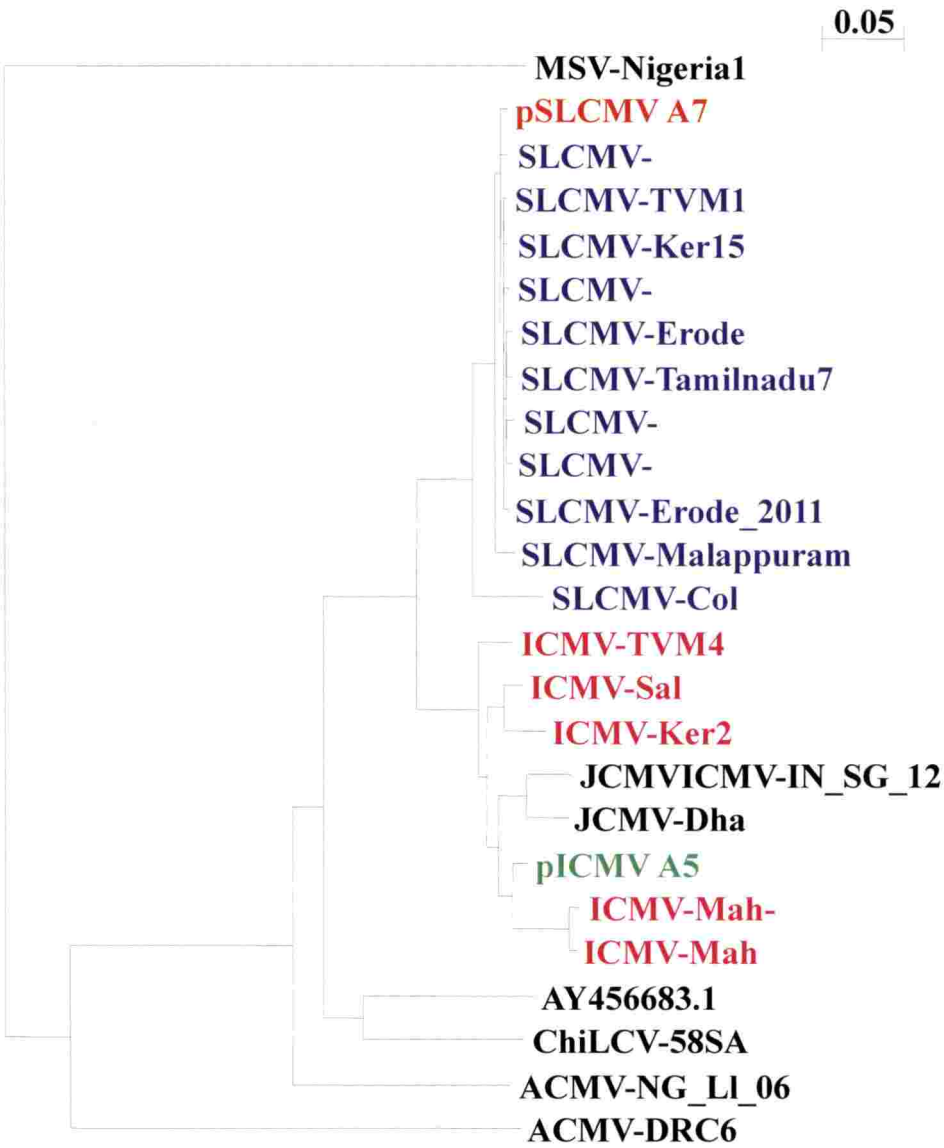


Fig. 19 Phylogram showing the relationship of SLCMV DNA A and ICMV DNA A isolates cloned in this study with previously reported cassava mosaic viruses. The phylogram was constructed using the neighbour-joining and bootstrap options of CLUSTALW

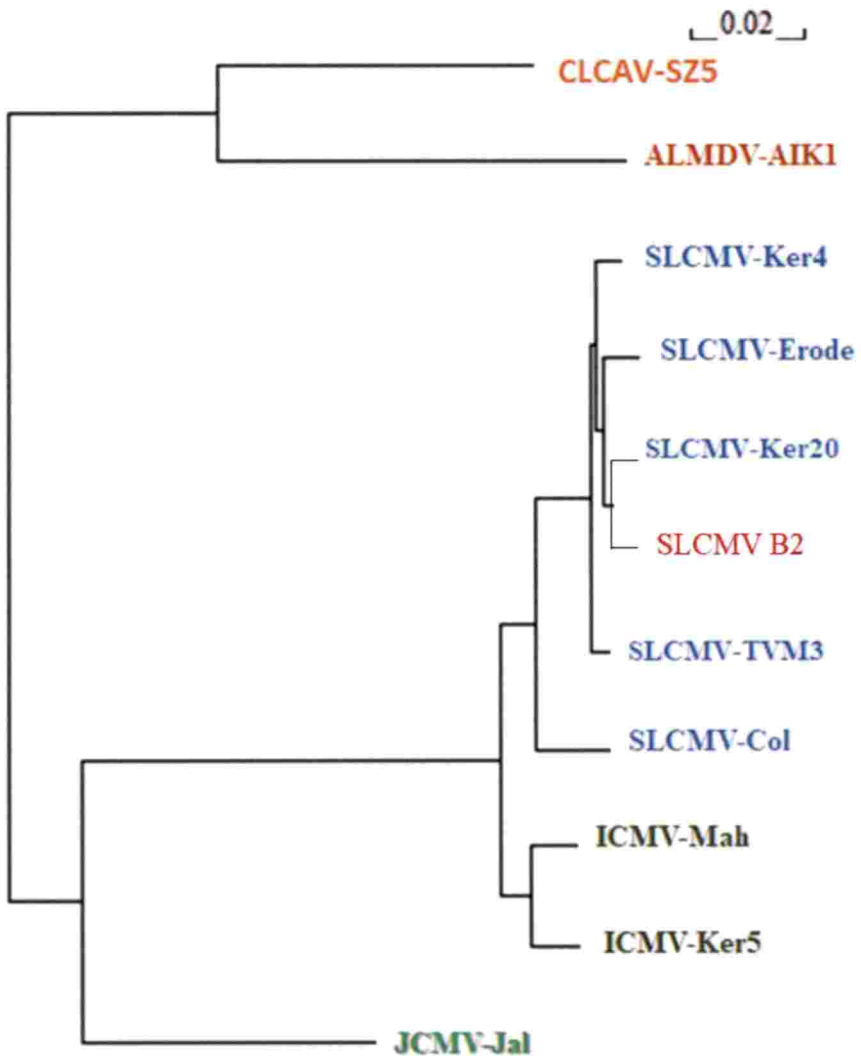


Fig. 24 Phylogram showing the relationship of SLCMV DNA B isolate cloned in this study with previously reported cassava mosaic viruses. The phylogram was constructed using the neighbour-joining and bootstrap options of CLUSTALW.

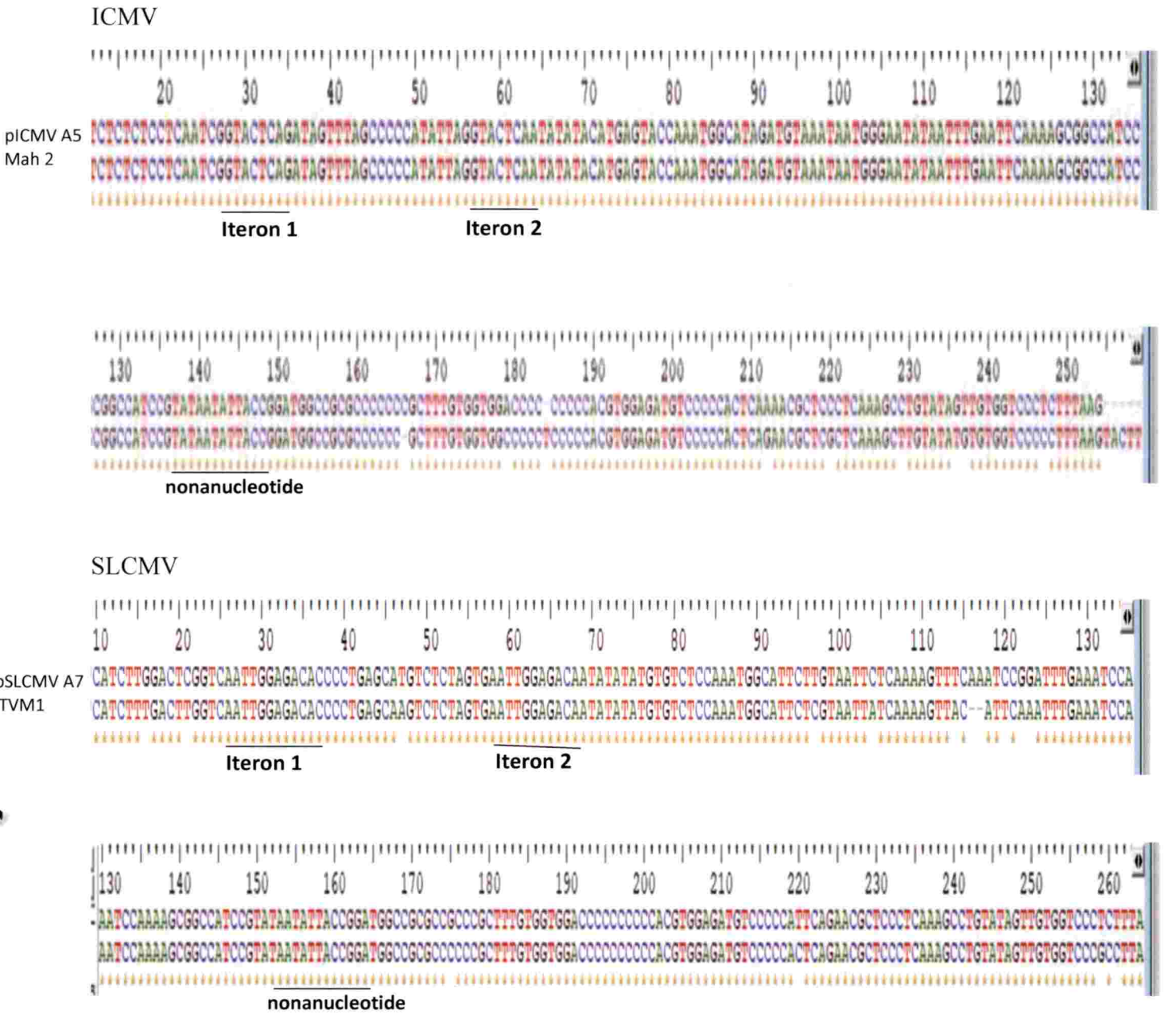


Fig. 20 Organisation of common region sequences of SLCMV A and ICMV A isolates. (a) Multiple sequence alignment of the common region (CR) sequences of pICMV A5 and ICMV Mah 2 clones showing positions of iterons (11-nt) and the nonanucleotide sequence. (b) Multiple sequence alignment of the common region (CR) sequences of pSLCMV A7 and SLCMV TVM 1 clones showing positions of iterons (11-nt) and the nonanucleotide sequence.

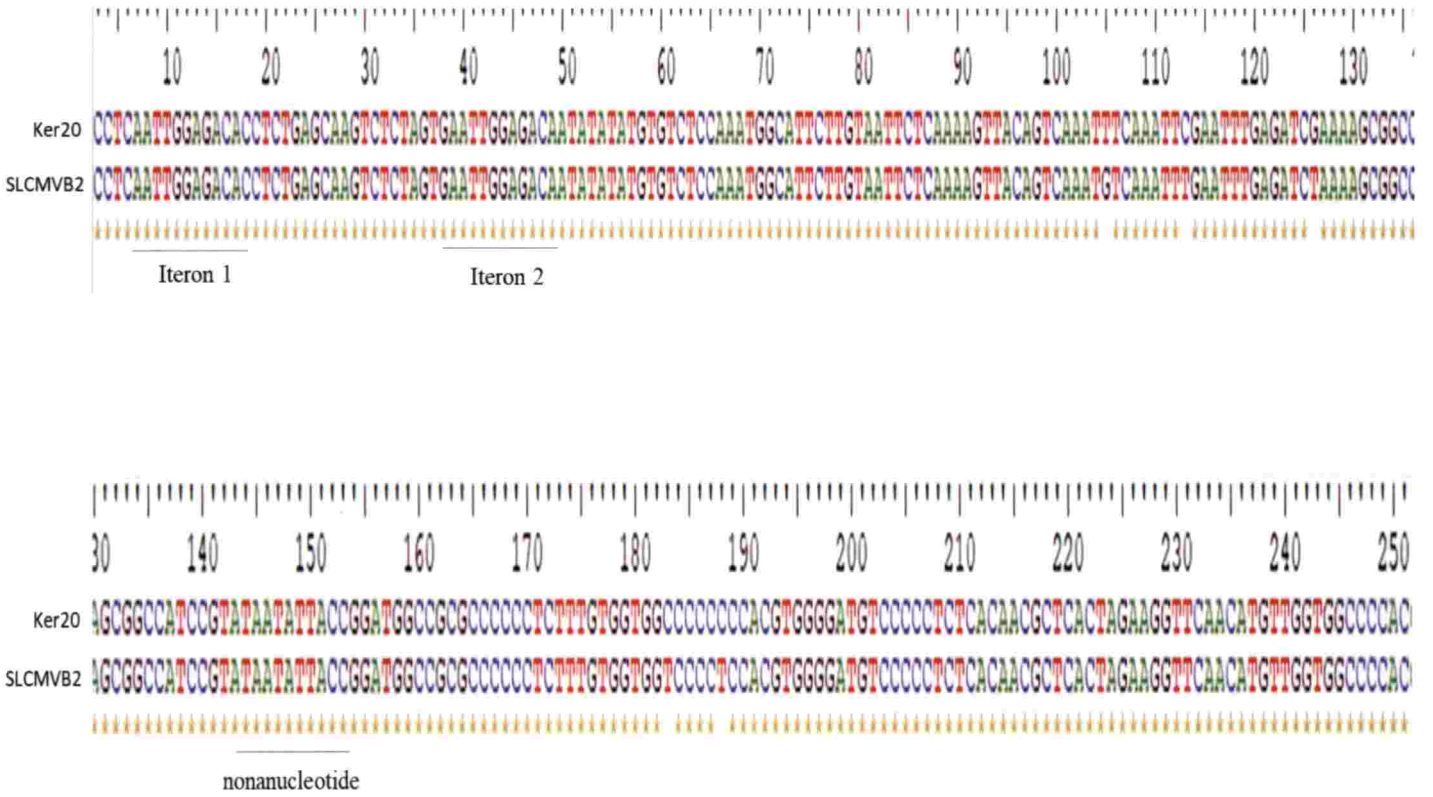


Fig. 25 Organisation of common region sequences of SLCMV B isolate. Multiple sequence alignment of the common region (CR) sequences of pSLCMV B2 and SLCMV Ker20 clones showing positions of iterons (11-nt) and the nonanucleotide sequence.

4.7 PARTIAL DIMER CONSTRUCTION OF SLCMV DNA A

4.7.1 Cloning of a 1.7 kb bitmer of DNA A from pSLCMV A7 into the binary vector pPZP201

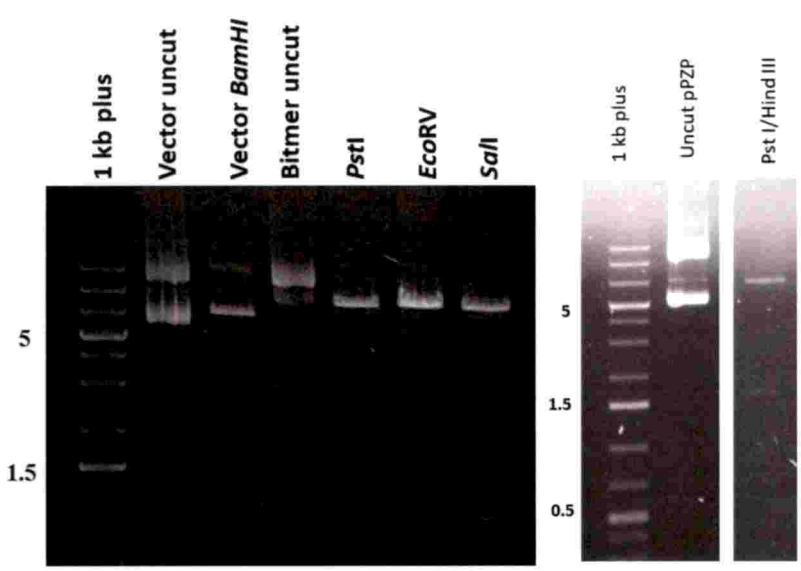
To obtain a 1.7 kb bitmer of DNA A, pSLCMV A7 was double digested with *PstI/Hin*dIII. Digestion with *PstI/Hin*dIII released three fragments of 1.7 kb, 1 kb and 2.7 kb. The 1.7 kb fragment was subcloned into the corresponding sites of the binary vector pPZP201. Screening was performed by blue/white selection. Cloning experiment yielded twelve blue colonies and five white colonies. For confirmation, five white colonies were taken for restriction analysis.

4.7.1.1 Confirmation of recombinant clones using restriction analysis

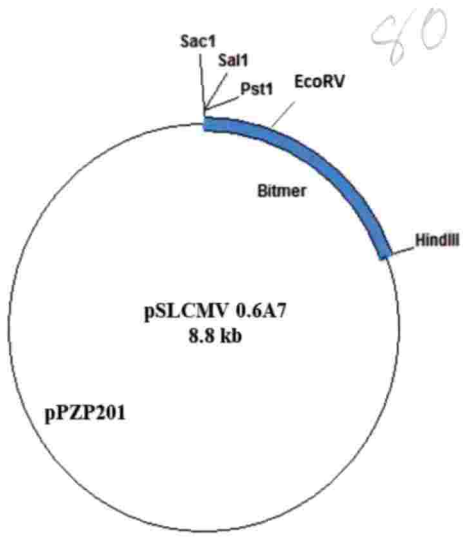
Size of the clone was expected to be 8.8 kb. To confirm that the five white colonies were clones, check digestion was performed with *Pst*I. Expected fragment to be released was 8.8 kb. Plasmid from two white colonies released the expected fragments. The clone was digested with multiple restriction enzymes such as *Eco*RV and *Sal*I. To release the 1.7 kb insert from the clone, double digestion was performed with *PstI/Hin*dIII. It was electrophoresed on a 0.8% agarose gel (Fig. 26 (a)). The sizes of the restriction fragments obtained are listed in Fig. 26 (b). The confirmed clone was named as pSLCMV 0.6A7 and a circular map was constructed (Fig. 26 (c)).

4.7.2 Cloning of a 2.7 kb onemer of SLCMV DNA A from pSLCMV A7 into pSLCMV 0.6A7

To construct one-and-a-bitmer (partial dimer) of DNA A, pSLCMV A7 was digested with *Pst*I. Two fragments were released; 2.7 kb full length DNA A and 2.7 kb of pUC19. The pSLCMV 0.6A7 vector was linearized using *Pst*I. Then both were ligated. The possibility of obtaining results were as follows (i) self-ligated vector, (ii) vector with 2.7 kb insert and (iii) vector with 2.7 kb pUC19 (iv) 2.7 kb insert with 2.7 kb pUC19. There was blue/white screening. The colonies were screened independently on two antibiotics. The colonies which grew on the



(a)



(b)

Enzymes	Sizes (kb) DNA A
PstI	8.8
SalI	8.8
EcoRV	8.8
PstI/HindIII	7.1+1.7

(c)

Fig. 26 Construction of pSLCMV 0.6A. (a) Restriction digestion pattern of pSLCMV 0.6A7. (b) Restriction map of pSLCMV 0.6A7 (c) The sizes of restriction fragments of pSLCMV 0.6A7

ampicillin + spectinomycin medium were considered as vector pUC19 clones. Colonies which did not grow on ampicillin but grew on spectinomycin were clones or self-ligated pPZP vector.

This experiment yielded about fifteen colonies on spectinomycin plates. Colonies were patched on spectinomycin + ampicillin medium. Eight patched colonies did not grow on ampicillin-containing medium. For clone confirmation, plasmid from 8 colonies was screened by restriction digestion with *Hin*dIII. An expected fragment of 2.7 kb was released from all the 8 clones.

4.7.2.1 Confirmation of recombinant clones using restriction analysis

Size of the clone was expected to be 11.4 kb. Multiple restriction enzyme analysis was performed with *Pst*I, *Hin*dIII, *Kpn*I, *Eco*RV *Sac*I and *Sal*I and electrophoresed in a 0.8 % agarose gel (Fig 27 (a)). Sizes of restriction fragments obtained are listed in (Fig. 27 (b)). The confirmed clone was named as pSLCMV 1.6A7 and the circular map was constructed (Fig. 27 (c)).

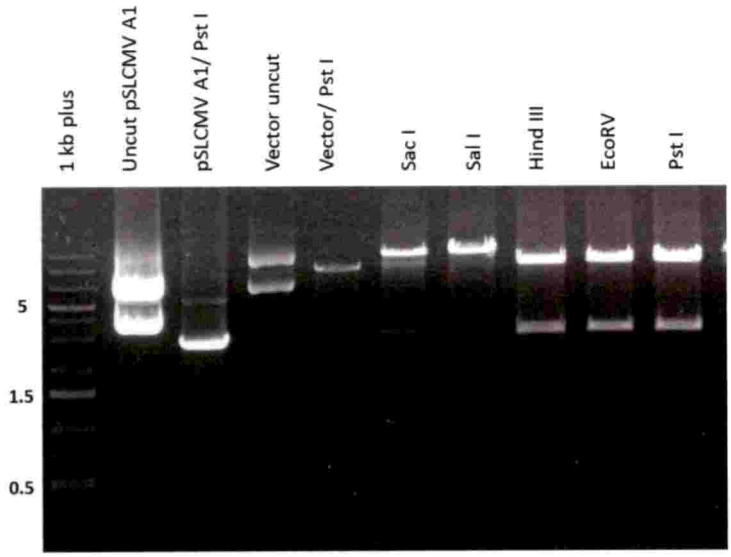
4.8 PARTIAL DIMER CONSTRUCTION OF SLCMV DNA B

4.8.1 Cloning of a 2.3 kb bitmer of DNA B from pSLCMV B2 into the binary vector pPZP201

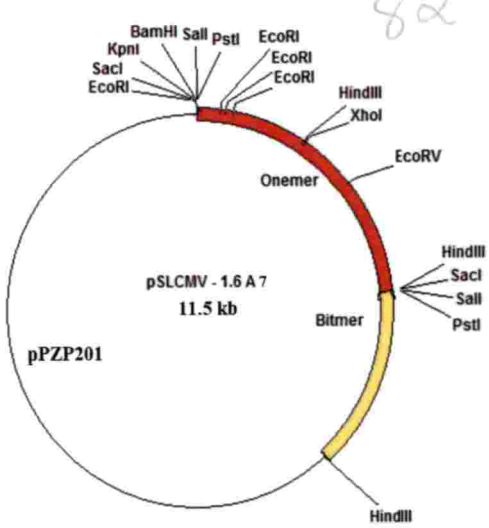
To obtain a 2.3 kb bitmer of DNA B, pSLCMV B2 was double digested with *Bam*HI/*Kpn*I. Digestion with *Bam*HI/*Kpn*I released three fragments of 2.3 kb, 0.4 kb and 2.7 kb. The 2.3 kb fragment was subcloned into the corresponding sites of the binary vector pPZP201. Screening was performed by blue/white selection. Cloning experiment yielded eight blue colonies and four white colonies. For confirmation, four white colonies were taken for restriction analysis.

4.8.1.1 Confirmation of recombinant clones using restriction analysis

Size of the clone was expected to be 9.4 kb. To confirm that the four white colonies were clones, check digestion was performed with *Hin*dIII. Expected fragment to be released were 8.1 kb and 1.3 kb. Plasmid from two white colonies



(a)



(b)

Enzymes	Sizes (kb) DNA A
SacI	11.4
SalI	11.4
HindIII	8.8+2.7
EcoRV	8.8+2.7
PstI	8.8+2.7

(c)

Fig. 27 Construction of pSLCMV 1.6A. (a) Restriction digestion pattern of pSLCMV 1.6A7. (b) Restriction map of pSLCMV 1.6A7 (c) The sizes of restriction fragments of pSLCMV 1.6A7.

released the expected fragments. The clone was digested with multiple restriction enzymes such as *Bam*HI, *Eco*RI, *Hin*dIII and *Kpn*I. To release the 2.3 kb insert from the clone, double digestion was performed with *Bam*HI and *Kpn*I. It was electrophoresed on a 0.8 % agarose gel (Fig 28 (a)). The sizes of the restriction fragments obtained are listed in Fig. 28 (b). The confirmed clone was named as pSLCMV 0.8B2 and a circular map was constructed (Fig. 28 (c)).

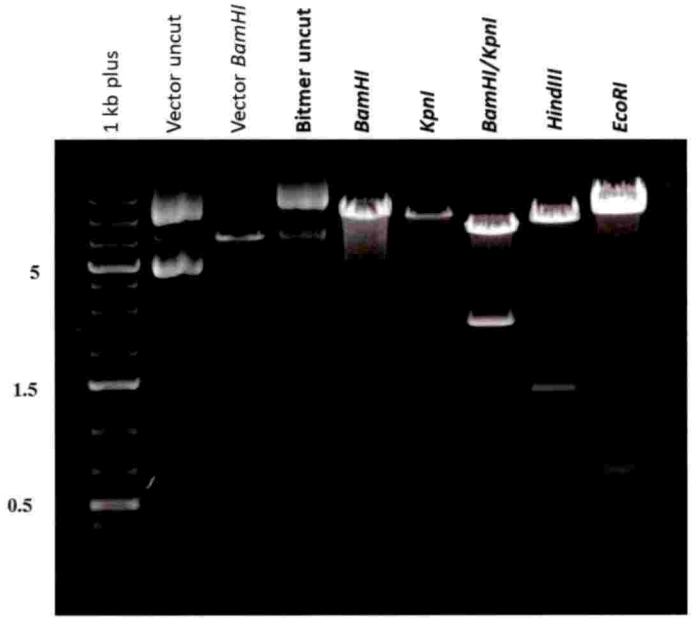
4.8.2 Cloning of a 2.7 kb onemer of SLCMV DNA B from pSLCMV B2 into pSLCMV 0.8B2

To construct one-and-a-bitmer (partial dimer) of DNA B, pSLCMV B2 was digested with *Bam*HI. Two fragments were released; 2.7 kb full length DNA B and 2.7 kb of pUC19. The pSLCMV 0.8B2 vector was linearized using *Bam*HI. Then both were ligated. The possibility of obtaining results were as follows (i) self-ligated vector, (ii) vector with 2.7 kb insert and (iii) vector with 2.7 kb pUC19 (iv) 2.7 kb insert with 2.7 kb pUC19 vector. There was blue/white screening. The colonies were screened independently on two antibiotics. The colonies which grew on the ampicillin + spectinomycin medium were considered as vector pUC19 clones. Colonies which did not grow on ampicillin but grew on spectinomycin were clones or self-ligated pPZP vector.

This experiment yielded about twenty colonies on spectinomycin plates. Colonies were patched on spectinomycin + ampicillin medium. Ten patched colonies did not grow on ampicillin-containing medium. For clone confirmation, plasmid from 10 colonies was screened by restriction digestion with *Kpn*I. An expected fragment of 2.7 kb was released from all the 10 clones.

4.8.2.1 Confirmation of recombinant clones using restriction analysis

Size of the clone was expected to be 12.1 kb. Multiple restriction enzyme analysis was performed with *Bam*HI, *Hin*dIII, *Kpn*I, *Sac*I and *Sal*I and electrophoresed in a 0.8 % agarose gel (Fig. 29 (a)). The clone was confirmed by double digestion with *Sac*I and *Sal*I released 5.0 kb (partial dimer) and 7.1 kb (pPZP201). Sizes of restriction fragments obtained are listed in (Fig. 29 (b)). The



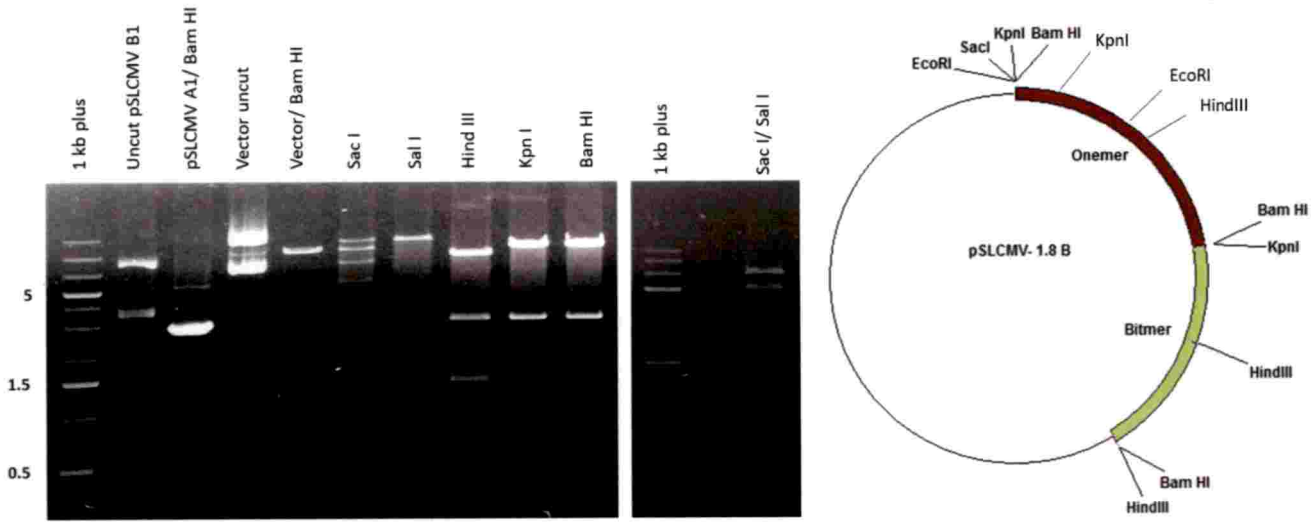
(a)

(b)

Enzymes	Sizes (kb) DNA B
BamHI	9.4
KpnI	9.4
BamHI/KpnI	7.1+2.3
HindIII	8.1+1.3
EcoRI	8.7+0.7

(c)

Fig. 28 Construction of pSLCMV 0.8B2. (a) Restriction digestion pattern of pSLCMV 0.8B2. (b) Restriction map of pSLCMV 0.8B2 (c) The sizes of restriction fragments of pSLCMV 0.8B2



(a) (b)

Enzymes	Sizes (kb) DNA B
SacI	12.1
SalI	12.1
HindIII	8.3+2.7+1.3
BamHI	9.4+2.7
KpnI	9.4+2.7
SacI/SalI	7.1+5.0

(c)

Fig. 29 Construction of pSLCMV 1.8B2. (a) Restriction digestion pattern of pSLCMV 1.8B2. (b) Restriction map of pSLCMV 1.8B2 (c) The sizes of restriction fragments of pSLCMV 1.8B2.

confirmed clone was named as pSLCMV 1.8B2 and the circular map was constructed (Fig. 29 (c)).

4.9 MOBILISATION OF PARTIAL DIMERS INTO *Agrobacterium tumefaciens* (*Ach5*)

Triparental mating was done. In the plate having 10^{-5} dilution, well defined colonies were seen in *Agrobacterium* strain containing both DNA-A and DNA-B. The plates are shown in Fig. 30.

4.10 CONFIRMATION OF TRANSCONJUGANTS

4.10.1 PCR

At first, these clones were confirmed for the presence of infectious clones through PCR with coat protein gene (CP) and movement protein (MP) primers specific to DNA-A and DNA-B components respectively (Fig 31 & 33). The PCR product for DNA-A specific primer was 1 kb while it was 1.1 kb band size for DNA-B specific primer.

4.10.2 Southern blotting

Sothern blot analysis was performed by using partial dimer of SLCMV DNA-A, linearized with *Sal*I as the probe. Two fragments of sizes 8.8 kb and 2.7 kb were expected to hybridize in the transformants upon *Pst*I digestion of total DNA from *Agrobacterium*. The expected fragments hybridized in all the transformants but not in the recipient strain (*Ach5*) (Fig. 32). This confirmed the presence of partial dimer of DNA A in the transformants.

In case of SLCMV B, southern blot analysis was performed using partial dimer of SLCMV DNA B, linearized with *Sal*I, as the probe. Two fragments of sizes 9.4 kb and 2.7 kb were expected to hybridize in the transformants upon *Bam*HI digestion of total DNA from *Agrobacterium* transformants. The expected fragments hybridized in all the transformants (Fig. 34) but not in the recipient strain (*Ach5*). This confirmed the presence of the partial dimer of DNA B in the transformants.

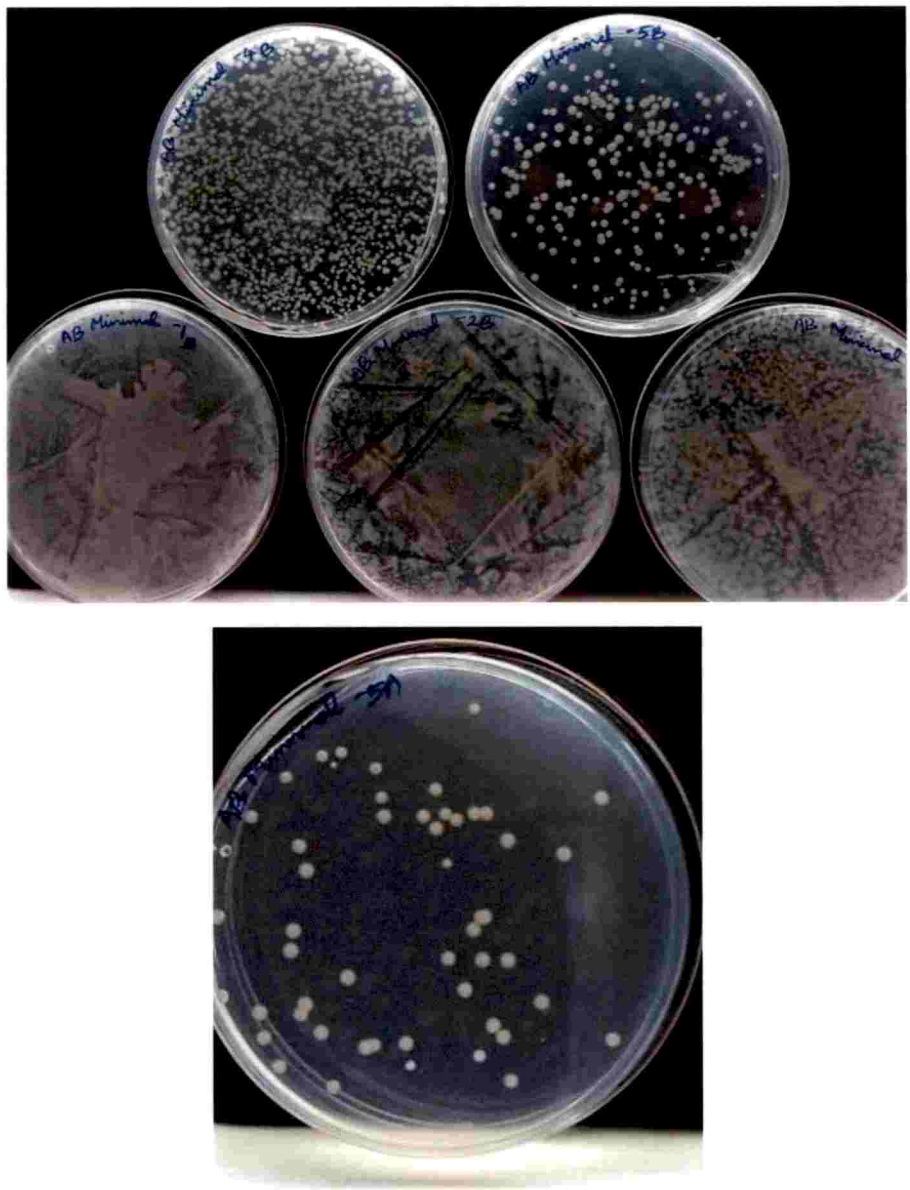


Fig. 30 AB minimal media plates showing the *Agrobacterium tumefaciens* (Ach5) colonies containing both DNA-A and B.

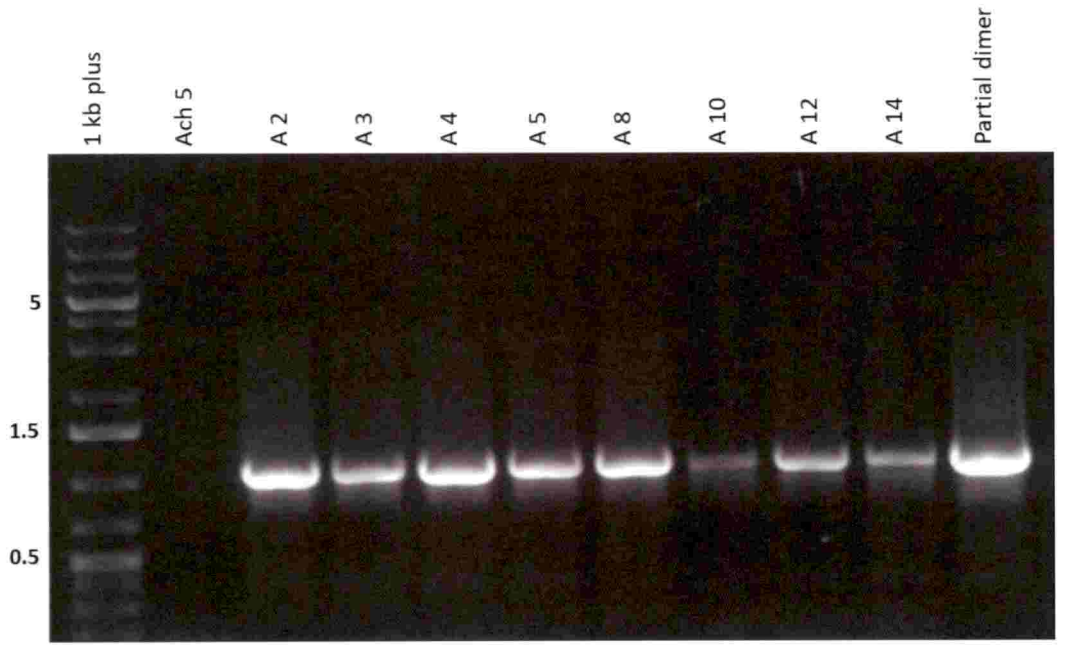


Fig. 31 PCR analysis of mobilisation of partial dimers of DNA A into *Agrobacterium* (PCR product- 1 kb)

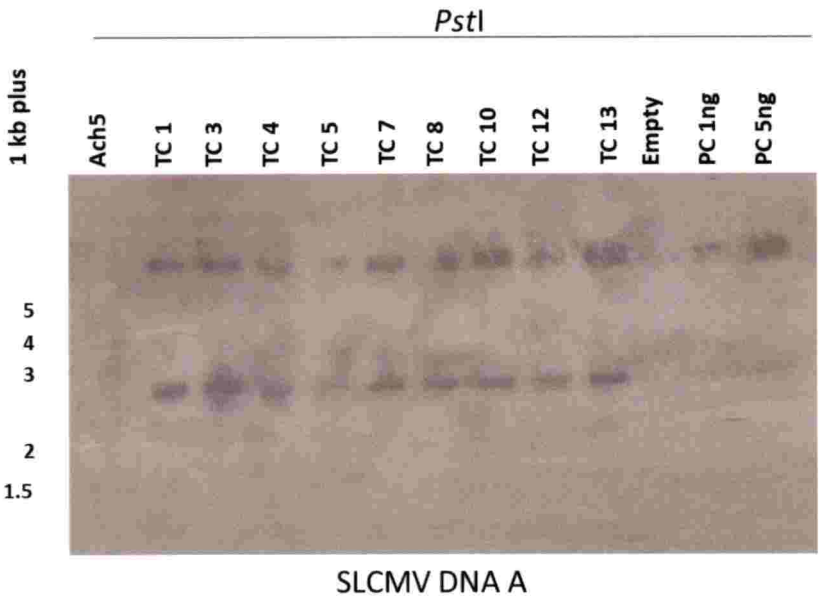


Fig. 32 Southern blot analysis of mobilisation of partial dimers of DNA A into *Agrobacterium.* Total DNA from eight transformants and the recipient *Ach5* was digested with *Pst*I and the blot was probed with DIG labelled pSLCMV 1.6A7. Fragments of sizes 8.8 kb, 2.7 kb hybridised in transformants and the positive control.

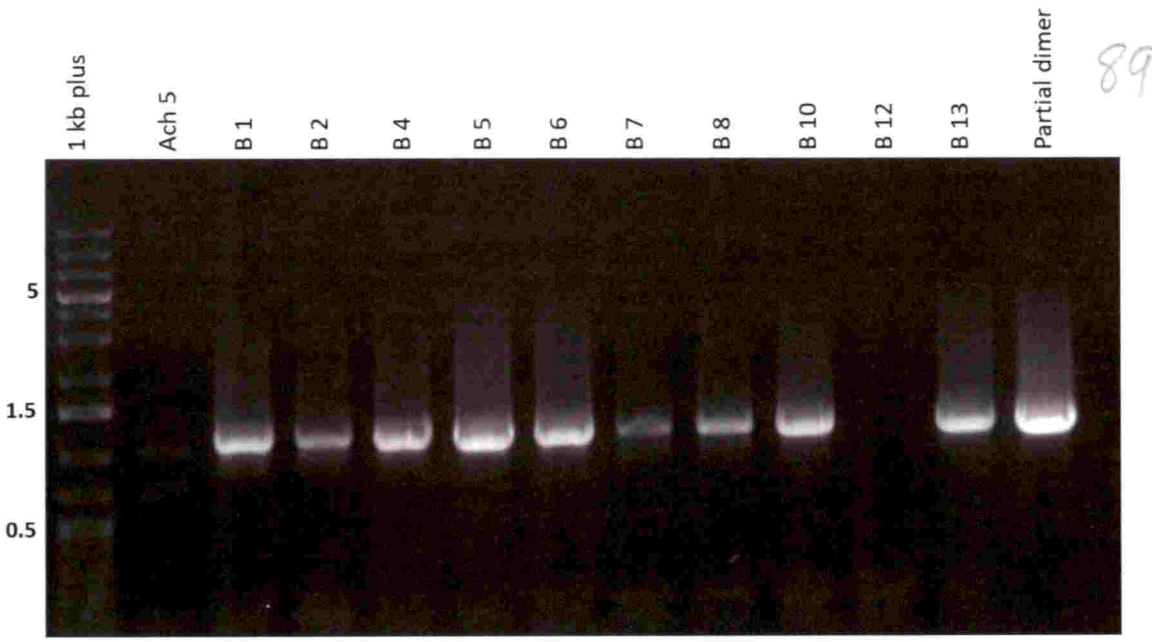


Fig. 33 PCR analysis of mobilisation of partial dimers of DNA B into *Agrobacterium* (PCR product- 1.1 kb)

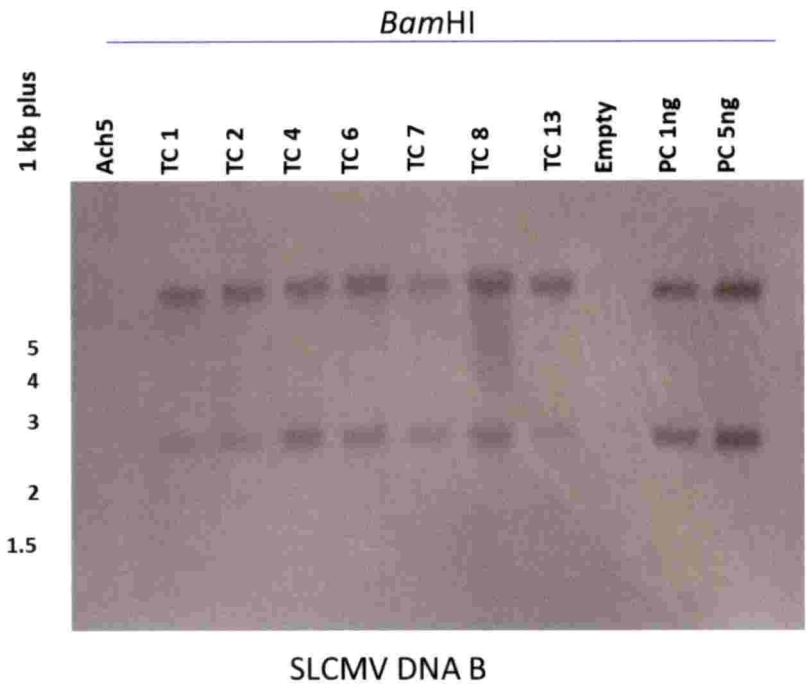


Fig. 34 Southern blot analysis of mobilisation of partial dimers of DNA B into *Agrobacterium.* Total DNA from eight transformants and the recipient Ach5 was digested with *Bam*HI and the blot was probed with DIG labelled pSLCMV 1.8B2. Fragments of sizes 9.4 kb, 2.7 kb hybridised in transformants and the positive control.

4.11 AGROINOCULATION

90

Nicotiana benthamiana plants with 5 to 6 leaves were injected with 20 µl bacterial suspension (in a phosphate buffer consisting of 100 µM acetosyringone) at the leaf internode using a fine needle. Control plants were inoculated with *Agrobacterium* recipient strain *Ach5*. *N. benthamiana* plants were inoculated with partial dimer of DNA A or DNA B alone and also with a combination of both DNA A and DNA B partial dimers.

Tumors developed at wounded sites of all the plants due to the *Agrobacterium* infection in one week. Plants inoculated with both DNA A and DNA B, initially showed mild stunting and downward leaf curling at 7 days of post inoculation (dpi). By 10 dpi five out of six plants displayed severe stunting, downward leaf curling and yellow mosaic pattern in the newly emerged leaves. Mild stunting, upward leaf curling and yellow vein phenotype was observed after two weeks of inoculation in the 4/5 plants infected with the partial dimer of DNA A alone. No symptoms were observed in the mock-inoculated control plants as well as plants inoculated with DNA B partial dimer alone (Table 9). A closer view of symptomatic leaves is presented in Fig. 35.

4.11.1 PCR analysis of agroinoculated *N. benthamiana* plants

Total DNA was extracted from the leaves of control plant, plants agroinoculated with DNA A or DNA B alone and symptomatic leaves infected with DNA A + DNA B. DNA sample were analysed by PCR using DNA_A- and DNA_B-specific primers.

4.11.1.1 PCR analysis using SLCMV DNA A-specific primer

Plant DNA (100 ng) was used as template and amplification was done with the positive and negative controls. The amplified products were electrophoresed on a 1 % agarose gel. DNA A primer pair yielded an amplicon of the expected size of 1 kb from the plants agroinoculated with the partial dimer of DNA A alone, DNA A + DNA B and also in the positive control, but not from the negative control

Table 9. Infectivity of *A. tumefaciens* containing partial dimers in *N. benthamiana*

INOCULUM	No. of plants inoculated	No. of plants showing symptoms	Symptoms
Ach5	3	0	~
DNA B	3	0	~
DNA A	5	4	Upward leaf curl
DNA A + DNA B	6	5	Stunting, leaf curl, chlorosis

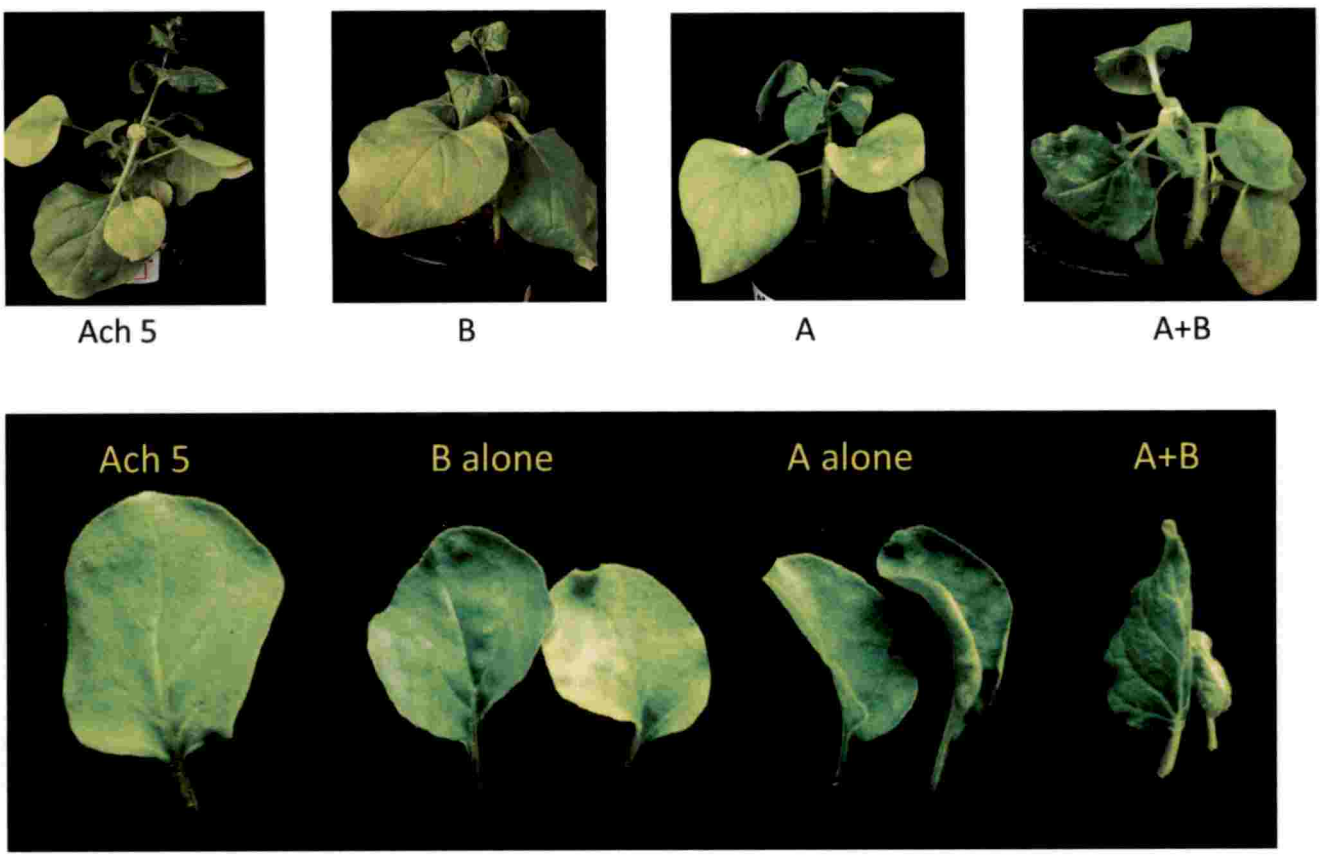
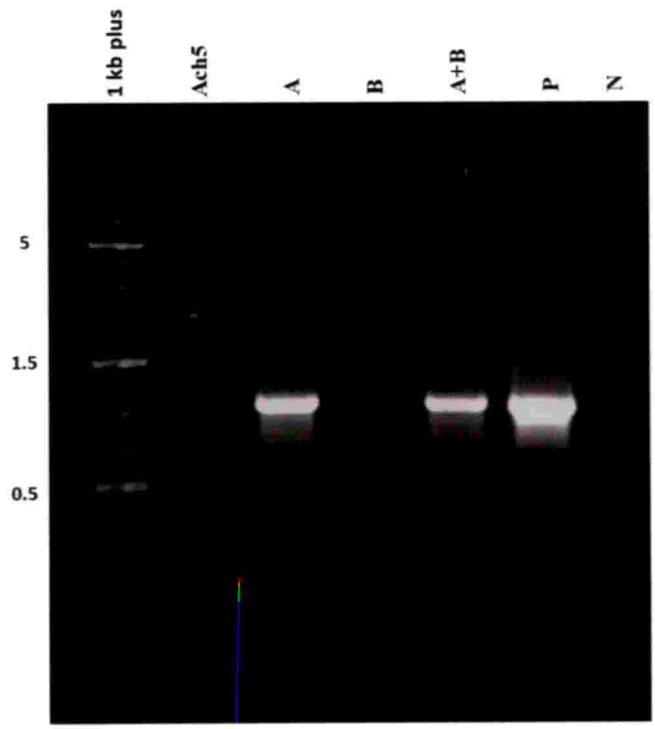


Fig. 35 Agroinfection- based infectivity analysis of pSLCMV A7 and pSLCMV B2 isolates in *N. benthamiana*. Leaves of *N. benthamiana* showing viral symptoms are shown.

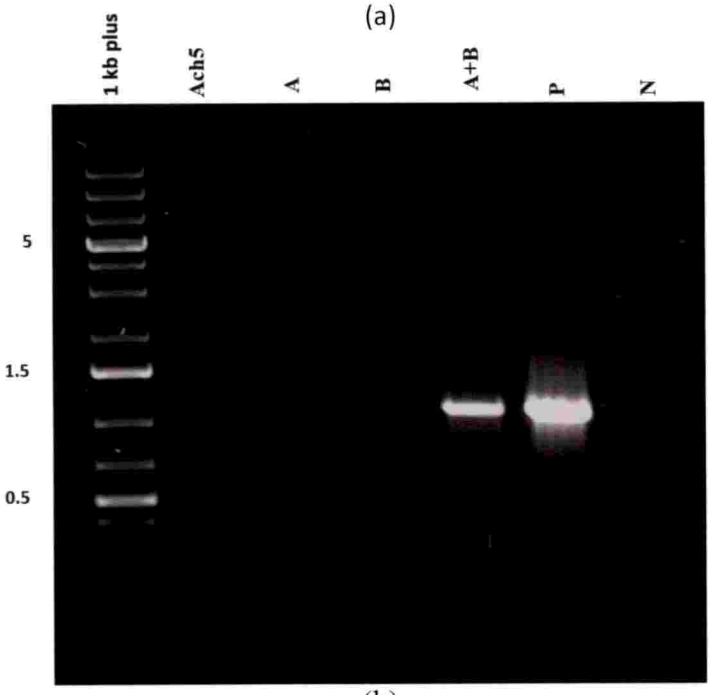
(Fig. 36 (a)). This confirmed that the symptoms are exhibited due to the infectious partial dimer clone of DNA A.

4.11.1.2 PCR analysis using SLCMV DNA B specific primer

Plant DNA (100 ng) was used as template along with the positive and negative controls. Amplification was done with DNA B-specific primers for the movement protein. The amplified products were electrophoresed on a 1 % agarose gel. Amplification was observed in the plants agroinoculated with both DNA A and DNA B and also in the positive control. The amplified product was found to be an expected size of 1.1 kb fragment (Fig. 36 (b)). No amplification was observed in the negative control and in plants agroinoculated with DNA B alone. This confirmed that the constructed partial dimer clones of DNA A and DNA B was infectious.



(a)



(b)

Fig. 36 PCR analysis of agroinfected plants(a) PCR analysis of DNA of agroinfected plants using DNA A pecific primer. A 1 kb band was obtained as PCR product (b) PCR analysis of DNA of agroinfected plants using DNA B pecific primer. A 1.1 kb band was obtained as PCR product

DISCUSSION

5. DISCUSSION

Cassava (*Manihot esculenta* Crantz, Family: Euphorbiaceae) is one of the most important tuber crops grown in the tropics and can be grown in a wide range of climatic conditions. Among the various diseases that affects the crop, cassava mosaic disease (CMD) is the major threat in cassava production. It is caused by cassava mosaic begomoviruses. Construction of infectious virus clones is an essential molecular tool for studying viral biological properties, validating the function of viral genes, and screening virus-resistant germplasm. So, development of efficient infectious clones of cassava mosaic virus and their subsequent inoculation technique would provide a major advancement to the resistance development in cassava.

PCR-based diagnosis is the best method to confirm the presence of cassava mosaic virus in the infected leaf samples. Among the various primers designed to detect these viruses, CP primer was found to be best (Makeshkumar *et al.,* 2005), but it cannot differentiate ICMV and SLCMV. So, in the present study, multiplex PCR (Dutt *et al.,* 2005; Manivasagam *et al.*, 2006; Makeshkumar *et al.*, 2007) was employed to differentially detect the presence of ICMV and SLCMV from infected samples.

Among the random leaf samples collected, only three samples had ICMV infection, while 25 samples had SLCMV infection. This reassured the view that SLCMV is widespread than ICMV in India (Patil *et al.*, 2005, Anitha *et al.,* 2011).

The whole genome amplification of SLCMV and ICMV was done by rolling circle amplification (RCA). The procedure was found to be very efficient, simple and highly sensitive as described by Haible *et al.* (2006). The ideal RCA products could be identified by digestion using appropriate restriction enzymes (Inoue-Nagata *et al.*, 2004).

In both SLCMV and ICMV clones, 200 nt common region has been identified that contains typical iterons and nonanucleotide sequence, TAATATTAC which constitutes the origin of replication (ori) (Fontes *et al.,*

1994; Stanley., 1995; Stenger *et al.*, 1991). Thus, rolling circle replication of these viral clones will happen even after agroinoculation, thus persistent infection begins in agroinoculated plants. Among the SLCMV and ICMV sequences, common regions exhibit less sequence homology while there is high sequence similarity outside their common region. Similar to the findings by Varsani *et al,* in 2004, DNA-A component of both SLCMV and ICMV isolates contain six genes *AV1, AV2, AC1*, *AC2*, *AC3* and *AC4,* while DNA-B component contains 2 genes, *BV1* and *BC1.*

The phlyogentic analysis of the DNA-A of both ICMV and SLCMV reveals that they are highly similar (96-99%) to the sequences of the corresponding species uploaded in the NCBI website. This evidence suggests low levels of genetic variability among the cassava mosaic virus as reported by Kushawaha *et al.* (2018). One possible reason behind the low variability of cassava mosaic viruses could be associated with the vegetative propagation of cassava practiced by farmers.

The sequence of pSLCMV A7 showed maximum similarity of 99 % with 'SLCMV-TVM1' sequence in NCBI blast. While the sequence of pSLCMV B2 showed maximum similarity of 99 % with 'SLCMV-Ker20 (Adivaram)' sequence in NCBI blast. The sequence of pICMV A5 showed maximum similarity of 95 % with 'ICMV- Mah' sequence in NCBI blast. It was also found that SLCMV DNA-A isolate is more closely related to ICMV DNA-A (84 %) than African cassava mosaic virus (ACMV) DNA-A (74 %).

The SLCMV DNA-A isolate showed similarity of 99 % with Kerala and Tamil Nadu isolates. While it showed only 93 % identity with Colombo isolate cloned from Sri Lanka. The SLCMV DNA-B showed 99 % identity with Kerala and Tamil Nadu isolates, 96 % identity with Colombo isolate cloned from Sri Lanka. This in turn shows that SLCMV-B component does not show high variation.

The success of the viral infection in certain plants depends on the mode of delivery of the nucleic acid. Of the three artificial methods used to introduce geminiviral DNA to plant hosts (mechanical rubbing, biolistic delivery and agroinoculation), agroinoculation was found to be a reliable and suitable method for artificial infection of plants with the viruses (Grimsley *et al.*, 1986). But agroinoculation in cassava plants is very difficult and only Mittal *et al.* (2008) got 60 % infectivity with SLCMV infectious clones. So, agroinfection of such clones are usually validated in *N. benthamiana* plants.

For the purpose of agroinfection the genomic components are subcloned as partial dimers and are transformed into a wild type *A. tumefaciens* strain, *Ach5* by triparental method. The use of tumorigenic wild type *A. tumefaciens* strain, *Ach5* is advantageous as tumor formation in plants is an indication for the efficient transfer of T-DNA into the plant cells (Grimsley *et al.*,1986). Also, Shivaprasad *et al,* in 2006 found out that *Agrobacterium* strains having C58 chromosome underwent deletion whereas no deletion occurred in strains *Ach5* and *T37*. Thus, it was concluded that chromosomal background of *A. tumefaciens* recipient strains is a very important factor in determining the deletion of viral sequences from partial dimers.

The partial tandem repeats containing two viral *ori* transformed into *Agrobacterium*, were agroinoculated in *N. benthamiana*. Due to the presence of two viral *ori* in the tandem repeats, a unit-length circular DNA is expected to be release by homologous recombination or by replicational release (Stenger *et al.*, 1991).

The transformants containing partial dimer of DNA-A usually undergo deletion of genomic-length viral DNA (2.7 kb) by recombination (Shivaprasad *et al.*, 2006). Therefore, it is important to carry out southern blotting analysis of *Agrobacterium* transformants to ensure that the partial dimers are intact in *Agrobacterium.* Southern blot analysis of nine *Agrobacterium* transformants with the SLCMV DNA-A partial dimer showed that the partial dimer was intact with both expected DNA fragments (8.8 kb and 2.7 kb) in all nine transformants.

Similarly, in all *Agrobacterium* transformants with the partial dimer of DNA-B, two fragments of sizes 9.4 kb and 2.7 kb were found in all seven transformants.

In the present study, partial dimers were cloned in pPZP binary vector having pVS1 replicon and so there was no deletion of the insert. This finding was same as that of Shivaprasad *et al*, in 2006.

Temperature is one of the most important factors for good viral spread (Burch-Smith *et al.*, 2004). So, the agroinoculated *N. benthamiana* plants were kept at 28^0C for obtaining best results. The symptoms of cassava mosaic disease came within 14 days post inoculation. Plants agroinoculated with partial dimer of DNA-A alone showed upward leaf curling which is typical for monopartite geminiviruses, suggesting the evolution of SLCMV from a monopartite geminivirus by the acquisition of DNA-B from ICMV (Saunders *et al.*, 2002). The DNA-A component has the ability to autonomously replicate and encapsidate in a permissive host in the absence of DNA-B in certain bipartite begomoviruses. This monopartite nature could be observed in three additional hosts, namely *N. tabacum,* Arabidopsis and cassava (Mittal *et al.,* 2008). DNA of ACMV and SLCMV alone systematically infected *N. benthamiana* when inoculated by agroinoculation (Klinkenberg and Stanley, 1990). The DNA-B cannot replicate in the absence of DNA-A (Klinkenberg and Stanley, 1990). Thus, in the present study, DNA-B of SLCMV did not cause any symptom. Agroinoculation of *N. benthamiana* with partial dimers of DNA-A + DNA-B of SLCMV caused a rapid appearance of symptoms. The symptoms included severe stunting, downward curling and yellow mosaic pattern on the newly emerged leaves. The PCR amplicons clearly showed that the virus has established itself in the host.

In the present study, one SLCMV DNA-A and its cognate DNA-B and one ICMV DNA-A has been cloned from field infected leaf samples. These clones were found to be infectious and will be useful for assays evaluating the resistance of transgenic cassava plants to geminiviruses.

SUMMARY

6. SUMMARY

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A study on "Development of infectious clones of cassava mosaic virus and their validation" was conducted at the ICAR- Central Tuber Crop Research Institute (ICAR-CTCRI), Sreekariyam, Thiruvananthapuram during 2017-2018. The important findings of the above studies are summarised in this chapter.

Out of the 39 samples randomly collected, 8 samples shown scale 1, 5 shown scale 2, 13 shown scale 3, 9 shown scale 4 and 4 samples shown scale 5 of very high mosaic symptoms having distorted leaves with stunting of plants.

Multiplex PCR could differentially detect the presence of ICMV and SLCMV from infected leaf samples. Among the 39 random leaf samples collected, only 3 samples had ICMV infection and 25 samples had SLCMV infection.

Using rolling circle amplification mechanism, whole genome of SLCMV DNA-A and its cognate DNA-B and ICMV DNA-A were amplified and cloned as pSLCMV A7 (2746 bp), pSLCMV B2 (2738 bp) and pICMV A5 (2739 bp) respectively, in pUC19 vectors. DNA B of ICMV clones were restricted using restriction enzymes such as *Eco*RI and *Hin*dIII and could not get the proper pattern, attempted further and unable to get the proper clone. The sequence of pSLCMV A7 showed maximum similarity of 99 % with 'SLCMV-[TVM1]' sequence in NCBI blast. While the sequence of pSLCMV B2 showed maximum similarity of 99 % with 'SLCMV-[Ker20]' sequence in NCBI blast. The sequence of pICMV A5 showed maximum similarity of 95 % with 'ICMV-[Mah]' sequence in NCBI blast.

Partial dimers were constructed for SLCMV DNA-A and SLCMV DNA-B. They were cloned in binary vector pPZP201. Binary vectors harbouring the partial dimers of SLCMV DNA-A and DNA-B were transformed into the wild type *A. tumefaciens Ach5* strain by triparental method. The transformants were confirmed by Southern hybridization.

Agroinoculation of *Nicotiana benthamiana* was done with the partial dimer constructs of DNA-A and DNA-B, in order to study the infectious nature of the clones. Agroinoculation of DNA-A + DNA-B partial dimers in *N. benthamiana* caused a rapid appearance of viral symptoms (downward leaf curling, stunting and yellow mosaic symptoms of leaves). Agroinfection of DNA-A partial dimer alone also caused the appearance of symptoms like upward leaf curling which is typical for monopartite geminiviruses. While plants infected with DNA-B alone did not cause any symptoms. The PCR amplicons clearly showed that the virus has established itself in the host.

These infectious clones of SLCMV were efficient and could be used for screening transgenic cassava plants for their level of resistance to cassava mosaic disease.

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APPENDICES

APPENDIX I

Reagents required for plant total DNA isolation

CTAB DNA Extraction Buffer

Tris HCl (pH 8.0)	:	100 mM
EDTA	:	20 mM
NaCl	:	1.4 M
CTAB	:	2 %
PVP	:	2 % (w/v)
β-mercaptoethanol	:	0.2 % (v/v)

(Freshly added prior to DNA extraction)

Autoclave and store at room temperature.

Chloroform-Isoamyl alcohol (24: 1)

Chloroform	:	24 ml
Isoamyl alcohol	:	1 ml

TE Buffer

Tris HCl (pH 8.0)	:	10 mM
EDTA	:	1 mM

APPENDIX II

Reagents required for Agarose gel electrophoresis

TAE Buffer (50X)

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5 M EDTA (pH 8.0)	:	100 ml

Gel loading dye (6X)

Bromophenol blue	:	25 mg
Xylene cyanol FF	:	25mg
Glycerol	:	3.3 ml
Distilled water	:	6.7 ml

APPENDIX III

Luria Agar Medium

35.0 g of LA (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature.

APPENDIX IV

LB Medium

20 g of LB (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

APPENDIX V

Preparation of LA Ampicillin /X gal /IPTG Plates

Ampicillin stock (50 mg/ml)

Dissolve 2.5 g Ampicillin in 50 ml deionized water. Filter sterilize and store at -20°C.

X gal stock (20 mg/ml)

Dissolve 0.2 g X gal in 10 ml N, N Dimethylformamide. Store at -20°C in the dark.

IPTG stock (100 mM)

Dissolve 1.7 g IPTG in 50 ml deionized water. Filter sterilize and store at 4°C in the dark

Before pouring the plates, allow the LA medium (1L) to cool to 55°C, then add 1ml ampicillin stock (making the final concentration to 50µg/ml). Mix gently and pour the plates. Dry the plates opened at room temperature under UV light for 30 min. Add 40µl X gal stock solution to the plate and spread evenly using a sterile spatula. Allow it to diffuse completely into the medium for about 10 min. Then add 40µl IPTG stock solution, spread evenly using a sterile spatula.

APPENDIX VI

Reagents required for plasmid isolation

Buffer P1

Tris (pH 8.0)	:	50 mM
EDTA (pH 8.0)	:	10 mM

Autoclave and store at 4°C

Buffer P2 (freshly prepared)

NaOH	:	200mM
SDS	:	1 %

Autoclave and store at room temperature

Buffer P3

Potassium acetate	:	3 M

Adjust the pH to 5.5 with glacial acetic acid. Do not autoclave. Use autoclaved distilled water. Store at 4°C.

APPENDIX VII

Reagents required for Agrobacterium mediated transformation

YEB medium

Beef extract	:	3g/L
Casein enzyme hydrolysate	:	5g/L

Yeast extract	:	1g/L
Sucrose	:	5g/L

Adjust the pH to 7.5 with 1 N HCl. Add 1.5% agar for making YEB agar. Autoclave and store at room temperature.

AB Buffer (20X)

Dibasic potassium phosphate	:	60g/L
Sodium dihydrogen phosphate	:	26g/L

The pH of both the solutions should be adjusted to 7. They have to prepared separately and mixed.

AB Salts (20X)

Ammonium chloride	:	20g/L
Magnesium sulphate	:	6g/L
Potassium chloride	:	3g/L
Calcium chloride	:	3.1g/L
Ferrous sulphate	:	0.05g/L

AB minimal medium (1 L)

Glucose or dextrose	:	5g/L
Agar	:	15g/L
AB buffer	:	50ml
AB salts	:	50ml

APPENDIX VIII

Reagents required for extraction of DNA from Agrobacterium tumefaciens

Lysis buffer

Tris acetate	:	400mM (pH 7.8)
Sodium acetate	:	20mM

EDTA	:	1mM
SDS	:	1%

APPENDIX IX

Reagents required for southern blotting

Denaturation solution

Sodium chloride	:	58.44g/L
Sodium hydroxide	:	20g/L

Dissolve the reagents in 800 ml distilled water and make up the volume to 1000 ml. Autoclave and store at room temperature.

Neutralisation solution

Sodium chloride	:	87.66g/L
Tris	:	30.56g/L

Dissolve the reagents in 800 ml distilled water, adjust the pH to 7 using 1N HCl and makeup the volume to 1000 ml. Autoclave and store at room temperature.

20X SSC

Sodium chloride	:	175.3/L
Sodium citrate	:	88.2g/L

Dissolve the reagents in 800 ml distilled water, adjust the pH to 7 using 1N HCl and makeup the volume to 1000 ml. Autoclave and store at room temperature.

Maleic acid buffer

Maleic acid	:	0.1 M
Sodium chloride	:	0.15 M

Adjust pH to 7.5 with conc. 1N NaOH. Autoclave and store at room temperature.

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Blocking solution (10%)

10 g of blocking powder in 100 ml maleic acid buffer. Dissolve by heating in microwave oven, autoclave and store at 4^0 C.

For 1% blocking solution, dilute with maleic acid buffer.

Pre-hybridisation solution

SSC	:	5X
N Lauryl sarcosine	:	0.1%
SDS	:	0.02%
Blocking solution	:	1%

Wash buffer

0.3% Tween 20 in maleic acid buffer. Do not autoclave.

ABSTRACT

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DEVELOPMENT OF INFECTIOUS CLONES OF CASSAVA MOSAIC VIRUS AND THEIR VALIDATION

VISHNU NARAYANAN

(2013-09-106)

Abstract of the thesis

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9. ABSTRACT

The study entitled "Development of infectious clones of cassava mosaic virus and their validation" was conducted at the ICAR- Central Tuber Crop Research Institute (ICAR-CTCRI), Sreekariyam, Thiruvananthapuram during 2017- 2018. The major objectives of the study were cloning and characterisation of SLCMV/ICMV infected leaf samples, construction of infectious clones of SLCMV/ICMV and agroinoculation of *Nicotiana benthaminana* with the partial dimers constructed in order to check the infectiousness of the viral clones. The whole genome amplification of SLCMV/ICMV DNA samples were done and cloned in pUC19 vectors to obtain pSLCMV A7 (2746 bp), pSLCMV B2 (2738 bp) and pICMV A5 (2739 bp) full length clones. The sequence of pSLCMV A7 showed maximum similarity of 99 % with 'SLCMV-[TVM1]' sequence in NCBI blast. While the sequence of pSLCMV B2 showed maximum similarity of 99 % with 'SLCMV-[Ker20]' sequence in NCBI blast. The sequence of pICMV A5 showed maximum similarity of 95 % with 'ICMV-[Mah]' sequence in NCBI blast. In order to develop infectious clones, partial dimers were constructed for SLCMV DNA-A and SLCMV DNA-B and cloned in binary vector pPZP201. These infectious clones were successfully transformed into wild type *A. tumefaciens, Ach5* strain by triparental method. Then agroinoculation of *N. benthamiana* with the constructed partial dimers was found to be successful. After 14 days post inoculation, plants infected with DNA-A + DNA-B partial dimers showed severe symptoms like leaf curling, stunting. The plants infected with partial dimer of DNA-A alone showed mild symptoms like upward leaf curling which confirmed its monopartite lineage. While those plants agroinoculated with partial dimer of DNA-B alone did not show any symptoms. These efficient infectious clones of cassava mosaic virus and their subsequent inoculation technique would provide a major advancement to the resistance development in cassava.